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# Proteomics Analysis of Rat Brain Postsynaptic Density

## IMPLICATIONS OF THE DIVERSE PROTEIN FUNCTIONAL GROUPS FOR THE INTEGRATION OF SYNAPTIC PHYSIOLOGY*

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**The postsynaptic density contains multiple protein complexes that together relay the presynaptic neurotransmitter input to the activation of the postsynaptic neuron. In the present study we took two independent proteome approaches for the characterization of the protein complement of the postsynaptic density, namely 1) two-dimensional gel electrophoresis separation of proteins in conjunction with mass spectrometry to identify the tryptic peptides of the protein spots and 2) isolation of the trypsin-digested sample that was labeled with isotope-coded affinity tag, followed by liquid chromatography-tandem mass spectrometry for the partial separation and identification of the peptides, respectively. Functional grouping of the identified proteins indicates that the postsynaptic density is a structurally and functionally complex organelle that may be involved in a broad range of synaptic activities. These proteins include the receptors and ion channels for glutamate neurotransmission, proteins for maintenance and modulation of synaptic architecture, sorting and trafficking of membrane proteins, generation of anaerobic energy, scaffolding and signaling, local protein synthesis, and correct protein folding and breakdown of synaptic proteins. Together, these results imply that the postsynaptic density may have the ability to function (semi-) autonomously and may direct various cellular functions in order to integrate synaptic physiology.**

The majority of excitatory neurotransmission in the brain occurs via glutamatergic synapses. In the presynaptic element of the synapse, specialized secretion machinery determines the activity-dependent membrane fusion of glutamate-containing vesicles and the release of transmitter into the synaptic cleft. In the postsynaptic element, glutamate receptors and downstream signal transduction are organized by the protein assembly of the postsynaptic density (PSD).[1] Both the presynaptic release machinery and the PSD are electron-dense assemblies (1, 2), in which proteins are thought to be organized into distinct functional complexes (3–5) that may be dynamically regulated by neuronal activity (6–8). The modulation of this molecular architecture of the synapse is at the basis of synaptic plasticity (6–8), and aberrations thereof may underlie neuronal disorders.

In view of the importance of the PSD in glutamatergic neurotransmission and its involvement in neuroplasticity, considerable efforts have been made to identify its protein constituents as a prelude to understand the molecular basis of PSD functioning. In the past several years, yeast two-hybrid technology has been extensively used to characterize proteins that interact with the glutamate receptors and may constitute core elements of the PSD involved in the regulation of receptor trafficking and signaling (reviewed in Refs. 9–11). Based largely on these studies a protein-protein interaction map of the PSD has emerged. In brief, the model posits that the postsynaptic receptor complexes are localized by scaffolding proteins such as the synapse-associated protein (SAP) 90 (also known as PSD-95). These proteins link the receptors to various signaling molecules *e.g.* nitric-oxide synthase, calcium/calmodulin-dependent protein kinase II (CaMKII), and inositol 1,4,5-trisphosphate receptors. Receptors and scaffolding proteins are further linked to cytoskeletal proteins, the arrangement of which will determine the morphology of and protein trafficking in the spine.

Yeast two-hybrid experiments are particularly useful in the detection of pairwise protein interactions, but are limited in their ability to reveal the global protein constituents of the protein complexes/organelles of interest. Alternatively, several PSD proteins have been identified from protein complexes by conventional biochemical means (12) or via the generation of

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[1] The abbreviations used are: PSD, postsynaptic density; CaMKII, calcium/calmodulin-dependent protein kinase II; CHIP, C terminus of HSP70 interaction protein; EF-1$\alpha$, eukaryotic elongation factor-1 $\alpha$; Hsp, heat shock protein; ICAT, isotope-coded affinity tag; IF-4, eukaryotic initiation factor-4; LC, liquid chromatography; MALDI-TOF/TOF® MS, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer; NMDA receptor, *N*-methyl-D-aspartic acid receptor; PMF, peptide mass fingerprint; SAP, synapse-associated protein; Ub-Pr, ubiquititation/proteasome; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid.

antibodies against the protein complex for screening of the expression library (13). For a global analysis of proteins the recently developed mass spectrometric-based proteome approach is particular attractive because hundreds of proteins can be displayed and identified. The large dataset generated can be used to formulate hypotheses and in turn design experiments to understand the (distinct) physiological processes carried out by the differentially composed protein complexes. Recently, several large scale proteome and proteomics studies have been reported (4–5, 14–18).

One of the first applications of proteome research in neuroscience was aimed at the characterization of novel PSD proteins (18). The PSD fraction was isolated using a standard protocol developed by Carlin *et al.* (19). The PSD proteins were partially separated on an SDS electrophoresis gel, trypsinized, and then characterized based on their peptide mass fingerprint (PMF). About thirty proteins, including "classic" and novel PSD proteins, were successfully elucidated. Overall, the number of proteins identified was lower than that expected, which may amount to above a hundred (4, 9–10). This suggested that a considerable number of PSD proteins remained to be characterized.

In this study we aimed at a much higher coverage of the PSD protein content in order to reveal groups of proteins that would predict novel synaptic functions. We employed two methods for the separation and detection of the proteins, namely (*a*) high-load preparative two-dimensional gel electrophoresis to separate a larger number of PSD proteins with increased resolution, and identified the tryptic peptides of individual protein spots by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer (MALDI TOF/TOF® MS), (*b*) isotope-coded affinity tagging (ICAT) of the proteins followed by trypsin digestion and the separation of cysteine-containing peptides by nano-liquid chromatography (LC) coupled online to an electrospray quadrupole-TOF mass spectrometer for the identification of the peptides (20). A large number of previously identified PSD proteins, as well as novel groups of proteins, were detected. Western blotting analysis of proteins from selected functional groups confirmed the enrichment of these proteins in the PSD. Grouping of the proteins based on their functions indicated protein complexes that are involved in diverse physiological activities, *e.g.* the receptors and ion channels, protein synthesis and breakdown, scaffolding, signal transduction etc. The wide diversity of proteins that may be active in the PSD clearly indicates the PSD to be the organizer of spine functioning sustaining the emerging view (21–22) that considers the dendritic spine as the smallest self-sustaining (semi)-autonomous neuronal organelle.

## EXPERIMENTAL PROCEDURES

*Purification of the PSD*—The PSD fraction was isolated either as described (23) based on the original method of Carlin *et al.* (19), or based largely on a variant of the original method (24). In brief, in the original method, forebrains of 30 days old untreated rats were homogenized in homogenization buffer (5 mM Hepes, pH 7.4; 320 mM sucrose) containing a protease inhibitor mixture (Roche Applied Science). Cell debris and nuclei were removed by 1,000 × *g* centrifugation. The supernatant was spun for 20 min at 12,000 × *g*, resulting in supernatant and pellet P2. The P2 pellet was further fractionated by centrifugation in a sucrose step gradient to obtain the synaptosome, an organelle that contains both pre- and postsynaptic compartments. Synaptosome was lysed in hypotonic solution to release the cytoplasmic proteins and organelles such as mitochondria and small synaptic vesicle, and the resulting synaptic membrane was recovered by centrifugation using the sucrose gradient as stated above. For isolation of the PSD fraction, the synaptic membrane was diluted with 12 mM Tris-HCl (pH 8.1), 320 mM sucrose and an equal volume of 1% Triton X-100, 320 mM sucrose. The suspension was stirred for 15 min and then centrifuged for 30 min at 33,000 × *g*. The pellet was resuspended in 320 mM sucrose, 0.5% Triton X-100, 5 mM Tris/HCl, pH 8.1. After 15 min of stirring, the PSD proteins were pelleted by a 2-h centrifugation at 201,800 × *g*. All steps were carried out at 4 °C. This PSD preparation was used for all the two-dimensional gel experiments.

In the variant method, the synaptic membrane was isolated as described above, and stirred for 30 min over ice in 1% Triton X-100 in 50 mM Hepes (pH 7.4). After centrifugation for 15 min at 30,000 × *g*, the pellet was suspended in 320 mM sucrose in Hepes buffer and loaded on top of a sucrose gradient consisting of 1 M, 1.5 M, and 2 M sucrose. The sample was centrifuged at 100,000 × *g* for 2 h, and the interface between 1.5 and 2 M was collected, mixed with equal volume of water containing 2% Triton X-100 and 150 mM KCl, and loaded directly on top of a sucrose gradient of 1.5/2 M. The gradient was centrifuged at 100,000 × *g* for 1 h. The interface at 1.5/2 M sucrose was collected, diluted 2× with water, and centrifuged to obtain the PSD fraction. After washing once with water, the pellet was redissolved in 0.5% SDS and then used for ICAT labeling, trypsin digestion, and liquid chromatography-tandem mass spectrometry studies.

*Two-dimensional Gel Electrophoresis*—Two-dimensional gel electrophoresis was carried out as described (25). In brief, samples were solubilized in lysis buffer for 30 min (9 M urea, 2% CHAPS, 20 mM Tris, pH 7.5, 0.5% dithiothreitol, and 0.5% IPG buffer 3–10) and then centrifuged. 370 μl of the supernatant was used for the rehydration and simultaneously loading of the proteins to the IPG strip (Immobiline 18 cm DryStrip 3–10 NL, Amersham Biosciences), at 30 V for 12 h. The voltage was increased to 8000 V and focused for a total of 65,000 V/hr. Immediately after being focused, IPG strips were wrapped in plastic foil and stored at −80 °C. Prior to SDS-PAGE, IPG strips were equilibrated in 6 M urea/2% SDS/1% dithiothreitol/50 mM Tris, pH 8.8/30% glycerol for 15 min, followed by equilibration in 6 M urea/2% SDS/2.5% iodoacetamide/50 mM Tris, pH 8.8/30% glycerol for 15 min. The second dimension separation was run overnight using the Isodalt System (Amersham Biosciences) in 1.5-mm 11% gels (Duracryl, Genomic Solutions) at 25 mA per gel at 15 °C. After electrophoresis, gels were fixed and stained using either silver or colloidal Coomassie Brilliant Blue G-250. The gels were washed once with water and stored at room temperature in a plastic sealing until tryptic digestion.

*Digestion of Proteins from Two-dimensional Gels*—All the visible protein spots from the Coomassie Blue-stained gel were manually excised with a round bottom dermal slicer of 3-mm diameter. The gel pieces were destained in 60% acetonitrile in 25 mM ammonium bicarbonate buffer, pH 8.5, and then dehydrated with 100% acetonitrile. The shrunken gel pieces were reswelled in 25 mM ammonium bicarbonate buffer, dehydrated again in 100% acetonitrile, and dried in a speedvac. For gel pieces that were heavily stained the rehydration/dehydration step was repeated once. The gel pieces were rehydrated in 8 μl of trypsin solution (20 μg/ml) for 1 h, followed by addition of 50 μl of 25 mM ammonium bicarbonate buffer to completely immerse the gel pieces. After incubation overnight at room temperature, 0.5 μl of the incubation buffer was pipetted to the MALDI plate and mixed with 1 μl of α-cyano-4-hydroxycinnamic acid. The samples were analyzed by a MALDI TOF/TOF® (Applied Biosystems) mass spectrometer (see below). In cases where the mass spectrometric signals were weak, the incubation buffer was loaded into a C18 Ziptip (Millipore) according to the protocol provided by the company. The bound peptides were eluted from the Ziptip using 1.0 μl of α-cyano-4-hydroxycinnamic acid, which was directly deposited onto the MALDI plate. The α-cyano-4-hydroxycinnamic acid matrix concentration was 5 mg/ml in 50% acetonitrile/50% water containing 0.1% trifluoroacetic acid.

For the identification of (the abundant) proteins from the silver stained gel, protein spots were digested as stated above. After incubation with trypsin the supernatant was loaded into a self-packed Poros R2 (PerSeptive Biosystems) micro-tip, and eluted in 5 μl 50% acetronitrile/1% formic acid directly into a spraying electrode and analyzed by an electrospray Q-TOF (Micromass) mass spectrometer as described previously (26) and below.

*Immunoblots*—P2, synaptosome, synaptic membrane and PSD fraction were lysed in two-dimensional gel electrophoresis lysis buffer, except that the IPG buffer and bromphenol blue were omitted from the lysis buffer. Protein concentrations were determined by Bradford assay. Equal volume of the extract was mixed with 2× SDS buffer, boiled for 2 min, and 10 μg was loaded to a 10% mini SDS-gel. Electrophoresis was carried out at 120 V for 1 h, and the proteins were electrotransblotted to nitrocellulose membrane in the CAPSO buffer containing 10% methanol at 30V overnight. The membranes were blocked in 2% bovine serum albumin in Tris-buffered saline for 1 h, incubated in the primary antibodies (1:1000) for 1 h, washed in Tris-buffered saline containing 0.05% Tween 20, and incubated in the secondary antibodies (1:2000) for another hour. Signals were devel-

oped by enhanced chemiluminescence (Amersham Biosciences). The primary antibodies were purchased from Upstate Biotechnologies (EF-1$\alpha$), BD Pharmingen (NMDA receptor subunit 1 clone 54.1), and Santa Cruz Biotechnology (sorting nexin 3). Anti PSD-95 was a gift from Dr. T. Südhof. The anti-ribosome serum was obtained from a patient with systemic Lupus erythematosus. All secondary antibodies were purchased from Dako.

*Mass Spectrometry of Trypsin-digested Protein Spots from Two-dimensional Gels*—The mass spectrometer utilized for the high throughput protein analysis was an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF™ Optics. This MALDI tandem mass spectrometer uses a 200 Hz frequency-tripled neodinium YAG laser operating at a wavelength of 355 nm. For MS/MS, ions generated by the MALDI process were accelerated at 8 kV through a grid at 6.7 kV into a short, linear, field-free drift region. In this region the ions passed through a timed-ion selector (TIS) device that is able to select one peptide from a mixture of peptides at different *m/z* for subsequent fragmentation in the collision cell. After a peptide at a given *m/z* was selected by the TIS it passed through a retarding lens where the ions were decelerated and then passed into the collision cell, which was operated at 7 kV. The collision energy was defined by the potential difference between the source and the collision cell and hence was 1 kV. Inside the collision cell the selected peptide ions collided with air at a pressure of $1 \times 10^{-6}$ Torr. After passing through the collision cell the ions (both intact peptide ion, the precursor, and fragments caused by collision with the air, the product ions) were reaccelerated in the second source region at 15 kV, passed through a second, field free, linear drift region, into the reflector and finally to the detector. The detector amplified and converted the signal to electrical current, which was observed and manipulated on a PC-based operating system. For reflector mode the operation of the instrument is far simpler. After the MALDI process generates the peptide ions they are accelerated at 20 kV through a grid at 14 kV into the first, short, linear, field-free drift region. After this point the rest of the instrument can be treated as a continuation of this field-free, drift region until the ions enter the reflector and then reach the detector where, as before, the signal at the detector is amplified and converted to electrical current. Both MS and MS/MS spectra were searched against the Mascot data base search engine (Matrix Science) to identify the proteins.

Electrospray (tandem) mass spectrometric experiments were performed on a Micromass Q-TOF mass spectrometer as described previously (26). The tryptic-digested samples were loaded into a nanoelectrospray capillary, which was pulled from a borosilicate glass capillary GC 100F-10 with a microcapillary puller. An internal wire electrode inserted inside the capillary was used for the measurement. The cone voltage was set at 25–30 V. For MS measurements, the quadrupole was operated in the rf-only mode and mass analysis was performed using the TOF analyzer. For tandem MS experiments, precursor ions were selected using the quadrupole, fragmented in the collision chamber using energies between 20 and 65 eV and argon as the collision gas, and the daughter ions detected by the TOF analyzer. Two major tryptic peptides were used for tandem MS, and the resulting daughter ion spectra were searched using the Mascot search engine. The electrospray MS that we used is less sensitive than the MALDI TOF/TOF® MS, and the potential co-migrating minor proteins in the protein spots are less likely to be detected.

*ICAT Labeling and Liquid Chromatography-Tandem Mass Spectrometry*—The PSD fraction of about 0.1 mg was dissolved in 200 $\mu$l of 0.5% SDS in 50 mM Tris buffer, pH 7.5. It was then diluted 2 times in 50 mM Tris buffer, incubated with 1 mM TCEP, and labeled with ICAT® reagents according to the instruction of the ICAT labeling procedure as provided by the company (Applied Biosystems) with minor variations. Briefly, after neutralization of the ICAT® reagents, the samples were further diluted to a final concentration of 0.05% SDS. Trypsin at a ratio of 1:10 with respect to the sample protein concentration was added and incubated overnight at 37 °C. The digest was acidified and loaded into a $4 \times 15$ mm cation exchange column equilibrated with 10 mM $KH_2PO_4$/25% acetonitrile, pH 3.0 at a flow rate of 1 ml/min. The column was washed with 3 column volumes of equilibration buffer to remove excess ICAT and other reagents and bound peptides were eluted from the column with 400 mM KCl in the same buffer and collected as single fraction. The fraction was neutralized and loaded into a $4 \times 15$ mm avidin column equilibrated in 2× phosphate-buffered saline. Non-ICAT-labeled peptides were removed by extensive washing with 2× phosphate-buffered saline, followed by washing with phosphate-buffered saline, 50 mM ammonium bicarbonate/20% methanol, pH 8.3 and water, and finally the bound ICAT-labeled peptides were eluted with 3 column volumes of 30% acetonitrile/0.4% trifluoroacetic acid. The eluted peptides were evaporated to dryness and reconstituted in 100 $\mu$l of cleavage reagent for 2 h at 37 °C to cleave the biotin portion of the tag from the labeled peptides. The fraction was dried, reconstituted in 150 $\mu$l of 5% acetonitrile/0.1% trifluoroacetic acid, and injected into a reversed phase capillary trap column at 40 $\mu$l/min. The peptides were resolved on a Dionex 75 $\mu$m × 15 cm C18 capillary column at 200 nl/min using a linear acetonitrile gradient from 5 to 50% in 60 min. The peptides were sprayed online into an electrospray quadrupole time-of-flight hybrid MS (QSTAR® from Applied Biosystems). Proteins were identified by analysis of collected MS/MS data using Pro ICAT™ software (Applied Biosystems).

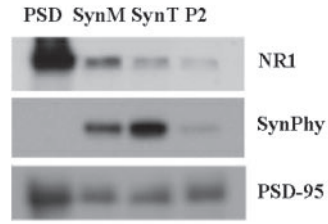


FIG. 1. **Immunoblots of NMDA receptor subunit 1, synaptophysin, and SAP90/PSD-95 from different cellular compartments.** Crude membrane pellet (*P2*), synaptosome (*SynT*), synaptic membrane (*SynM*), and PSD fraction (*PSD*) were dissolved in lysis buffer, mixed with 2× SDS buffer, and 10 $\mu$g each were resolved in a 10% SDS gel. After electroblotting to nitrocellulose membranes, the proteins were visualized using antibodies against NMDA receptor subunit 1 (*NR1*), synaptophysin (*SynPhy*), and SAP90/PSD-95 (PSD-95), respectively.

## RESULTS

For two decades the 2× Triton extraction method to yield the PSD fraction from rat brain as developed by Carlin and coworkers (19, 24) has been the standard protocol for the purification of PSD proteins and has been used in previous studies as the source for the characterization of novel PSD protein components. In the present study two independent proteome approaches were used, namely two-dimensional gel electrophoresis in conjunction with MALDI tandem mass spectrometry, and the ICAT peptide derivatization technique with nano-liquid chromatography coupled online to electrospray tandem mass spectrometry.

In the first instance we used the two-dimensional gel-based method for the isolation of the PSD proteins. We verified the purification efficacy of the protocol resulting in the PSD fraction. We then completed the characterization of the proteins from the PSD fraction.

First, we examined the enrichment of well-established PSD proteins in the PSD fraction, namely an NMDA receptor subunit NR1 and SAP90/PSD-95, and the depletion of a presynaptic marker protein, the integral synaptic vesicle membrane protein synaptophysin. Equal amounts of P2, synaptosome, synaptic membrane, and PSD fractions were run on an SDS gel, electroblotted onto nitrocellulose membranes and immunostained with antibodies against NR 1, SAP90/PSD-95, and synaptophysin, respectively. Fig. 1 reveals the high enrichment of the NR1 and SAP90/PSD-95 in the PSD fraction. Synaptophysin is enriched in the synaptosome, diminished in the synaptic membrane and totally absent from the PSD fraction.

Second, the progress of purification was monitored by two-dimensional gel electrophoresis. 400-$\mu$g protein extracts of synaptosome, synaptic membrane, and the PSD fractions were separated by large format two-dimensional gels and stained with silver. The synaptosome fraction contains a high number of protein species over a wide range of isoelectric focusing point (pI) and $M_r$ (Fig. 2*A*). The PSD fraction on the other hand exhibits a less complex protein pattern (Fig. 2*C*). Compared with the synaptic membrane a number of protein spots are relatively reduced in the PSD fraction, indicating that these

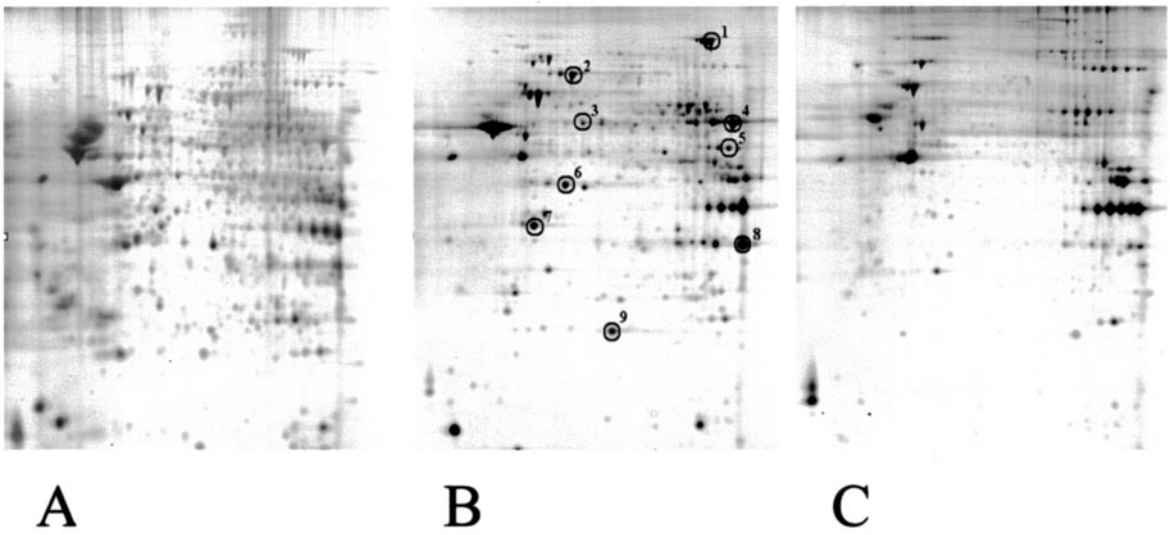


FIG. 2. **Typical examples of the protein profiles of the synaptosome (*A*), the synaptic membrane (*B*), and the PSD fraction (*C*), separated by two-dimensional gel electrophoresis and stained using silver.** The *circled protein spots* in *B* were chosen for further characterization by tandem MS because they are highly expressed in synaptic membrane but are greatly diminished in the PSD fraction, indicating that they may not anchor to the PSD. *1*, aconitase; *2*, dihydrolipoamide acetyltransferase; *3*, dihydrolipoamide succinyltransferase; *4*, ATP synthase $\alpha$ chain; *5*, fumarase; *6*, isocitrate dehydrogenase; *7*, pyruvate dehydrogenase; *8*, voltage-dependent anion-selective channel; *9*, H-ATP synthase subunit d.

proteins may not anchor to the PSD core (Fig. 2*B*). We then characterized nine of these proteins spots that are highly expressed in the synaptic membrane but apparently are absent or greatly reduced in the PSD fraction. Electrospray tandem mass spectrometry reveals that eight of them are mitochondrial proteins, namely aconitase, dihydrolipoamide succinyltransferase, ATP synthase $\alpha$ chain, H-ATP synthase subunit d, pyruvate dehydrogenase, isocitrate dehydrogenase, fumarase, and dihydrolipoamide acetyltranstransferase. Furthermore, there is a single glial cell-specific protein, the voltage-dependent anion-selective channel protein 1. Six of these proteins are not detectable in the PSD fraction (see Table I), and the other three proteins appear as minor spots. This shows that the Triton X-100 extraction step selectively removes proteins that are known to be exogenous to the PSD. But it also indicates that a small fraction of highly abundant proteins, notably mitochondrial and glial proteins, might survive the detergent extraction step and a minor fraction of them partitions into the PSD fraction.

Although the comparison of the protein profiles across the two-dimensional gels provides information about the probable enrichment of specific proteins in the PSD fraction, for two reasons we did not use this as the criterion to select spots for identification. First, distinct proteins may have similar physical properties that may co-migrate in the two-dimensional gel, in which case the apparent "diminished" spots in the PSD fraction may still contain "genuine" PSD proteins that are masked by a more abundant non-PSD protein in the synaptic membrane. Indeed, even some of the protein spots of the less protein-dense PSD gel contained multiple protein species (see Table I). Second, the protein profiles of the synaptic membrane and the PSD fraction have considerable differences in protein content, which makes the chance of mismatching significant. Taken together, we opted for the characterization of all proteins of the PSD fraction that were displayed on the two-dimensional gel.

It is generally known that silver-stained protein spots are less favorable for subsequent mass spectrometric analysis, and give considerable lower yield and coverage compared with those stained with Coomassie Blue. On the other hand, the detection limit of colloidal Coomassie Blue staining is about 5–10 times higher than silver staining. To compensate for the lower sensitivity of the Coomassie Blue staining we increased the loading of PSD proteins to the two-dimensional gel by about 7 times (*i.e.* 3 mg of protein), which yield a protein profile that is approximately equal to that of silver stained gel.

All together, 250 protein spots are visible to the bare eye (Fig. 3). These spots were excised and digested with trypsin. The trypsin autodigestion fragments at the protonated masses of 842.510 Da and 221.104 Da, were used as internal standards for the calibration of the MS spectra. The MS resolution for the peptides was generally greater than 10,000 full-width half-maximum, and the mass accuracy better than 10 ppm. For the data base search the mass tolerance was set at 0.03 Da. The MS/MS resolution was ~3000–6000 full-width half-maximum. No internal standard was used for calibration and the mass tolerance was set higher at 0.3 Da although mass error was expected to be less than 0.1 Da. All the mass spectra were searched against the NCBI data base, using online mascot software.

The identification of the proteins is based on several criteria. The most important criterion is the mascot score of the PMF, and in most cases the scores are significant. Further, we also consider the mascot scores of the daughter ion spectra of the tryptic peptides, and the matching of the identified proteins to the calculated $M_r$ and pI. Often, single proteins are present as several protein spots. As such, we are able to (tentatively) assign the identities of several proteins that have low mascot scores by taken into account of the fact that the next nearest spot differs only slightly in pI and probably also contains the same protein.

Proteins contained in most of the spots are identified (Table I). The mitochondrial and glial cell-specific proteins are known to be the contaminants. All other proteins are considered as potential PSD proteins, unless otherwise stated (Table I). These proteins are clustered into distinct groups based on their described functions (Table II) that can be assigned to diverse physiological activities, including the cytoskeleton and their interacting proteins for the maintenance and modulation of synaptic architecture, the proteins involved in sorting and trafficking of membrane proteins, the proteins involved in anaerobic energy supply for the acute requirement of energy during

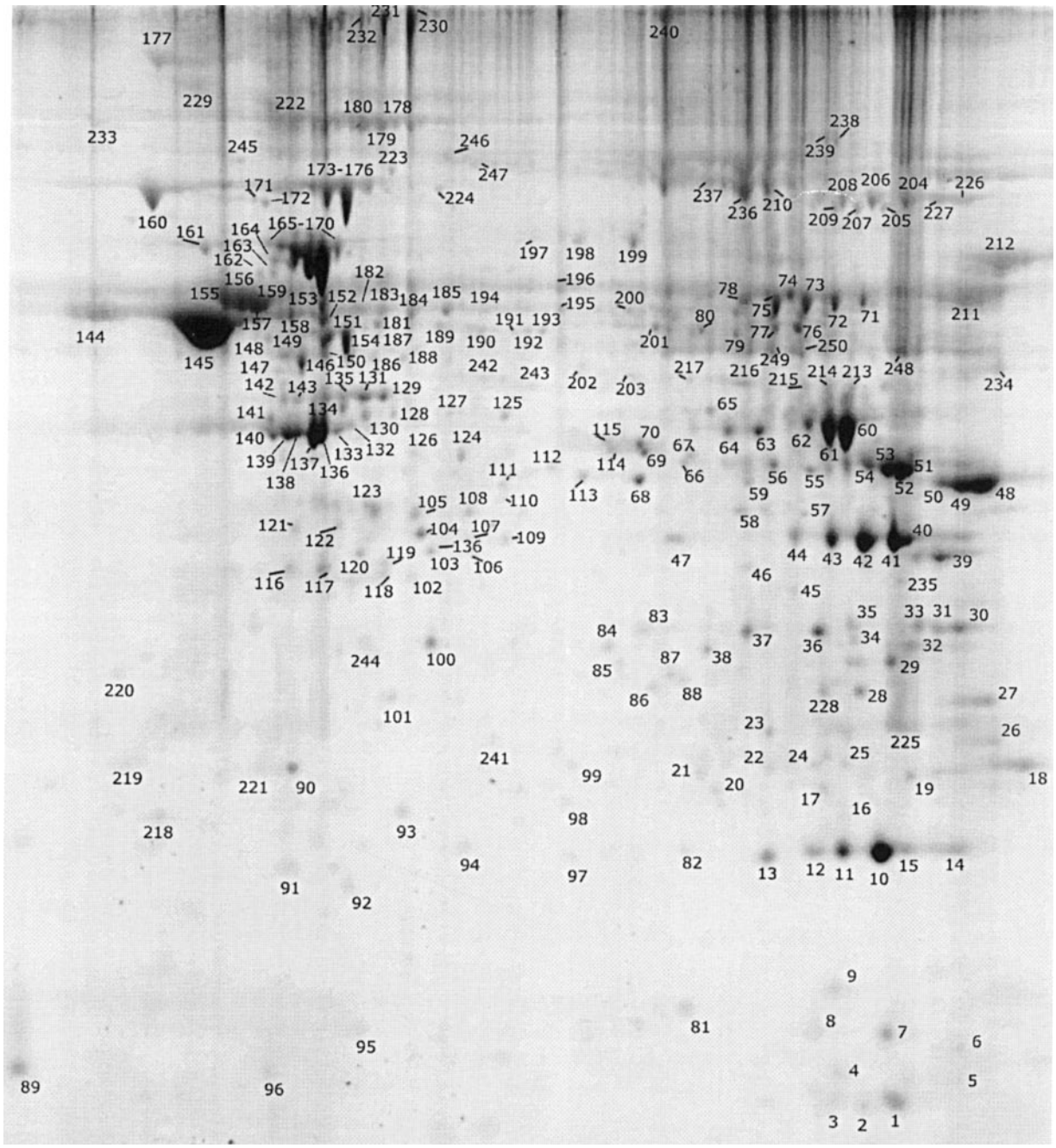

FIG. 3. **Two-dimensional separation of 3 mg of PSD protein stained with colloidal Coomassie Blue.** All visible spots were labeled from 1–250, cut out, and tryptic-digested followed by MALDI MS and MS/MS analyses. There are a number of spots that are not visible to the bare eye but can be detected by increasing the contrast of the computer image. These spots could not be picked manually and therefore were not analyzed in the present study.

intense neuronal activity, the proteasome system for specific synaptic protein degradation, the chaperone system for correct protein folding, the local protein synthetic machinery, and the scaffold and signaling protein complexes. Finally there are also a number of novel proteins and proteins with described functions that as yet cannot be assigned in the context of PSD functioning. Nevertheless, these proteins may be of functional significance. For example, the primary sequence of the hypothetical 18.5 kDa protein (spot number 9, Table I) is highly conserved between mouse and human suggesting that it may serve an important function.

Among the proteins of the PSD fraction some of them may be localized exclusively to the PSD and are therefore highly enriched in the PSD with respect to other cellular compartments, *e.g.* the NMDA receptors and SAP90/PSD-95. Alternatively, some proteins may be present in multiple cellular compartments including PSD. In the latter case their enrichment in the PSD would be less pronounced than for PSD-specific proteins. To gain better insight into this compartmental localization of proteins from distinct functional classes, we performed some initial immunoblot experiments. Three proteins were chosen based on the assumption that they may also be present in other compartments than the PSD, namely EF-1$\alpha$, a translational factor; sorting nexin 3, a protein that is involved in the trafficking of membrane proteins; and ribosomal protein. Interestingly, Fig. 4 reveals that these three proteins are selectively enriched in the PSD fraction, indicating that they may be tightly anchored to the PSD core.

Two-dimensional gel electrophoresis-based methodology is a widely used technique for proteomics studies. However, in our

TABLE I

*Mass spectrometric characterization of PSD proteins fractionated by two-dimensional gel electrophoresis*

The spot numbers (Spotnr) correspond to the protein spot numbers indicated in Fig. 3. Mass spectra were searched against NCBI database using mascot software from Matrix Science. For all samples the tryptic peptide mass fingerprints (PMF) were searched, and their scores listed under direct analysis mascot score PMF. The six most intense peptide peaks from the tryptic peptide fragments of the proteins were automatically selected for MS/MS analyses, and the mascot scores of the daughter ion spectra are listed under MS/MS. Blank spaces in the mascot score mean no hit, which are the cases when the mascot scores are low and the measured and the predicted $M_r$ and pI of the proteins do not agree. Often, a series of spots that differ slightly in *pI* represent the same proteins. Accordingly, some of the proteins that have a relatively low score but are positioned within such a series that match the predicted $M_r$ and *pI* are also listed. Some samples were further processed with a Ziptip, and their mascot scores are listed under Ziptip mascot score. The mitochondrial and glial cell-specific proteins can be considered as contaminants and are shown in italics. CDC rel-1AI and peanut (Drosophilia)-like 1 differ at the C-terminus; the last 5 amino acids in CDC rel-1AI are replaced by another 21 amino acids in peanut (Drosophilia)-like 1. Peanut (Drosophilia)-like 1 is positively identified since the peptide fragment contained in the C-terminal region is detected from the mass spectrum. In cases where the fragment from the C terminus was not detected, the proteins are labelled as CDC rel-1AI. Based on the fact that peanut (Drosophilia)-like 1 is slightly bigger than CDC rel-1AI, spots numbered 70 and 115 most likely contain peanut (Drosophilia)-like 1 whereas spots numbered 69 and 114 CDC rel-1AI. Spots numbered 130 and 134 contained a protein with PMF and MS/MS data that corresponds to rat neurofilament triplet M protein. The measured $M_r$ on the two-dimensional gel, however, is about 40 kDa lower than expected. This protein may represent a fragment, as is the case for pig neurofilament triplet M protein. Full-length rat neurofilament triple M protein is also present, *e.g.,* fraction 233. Glyceraldehyde-3-phosphate dehydrogenase exists as two groups; a group (numbered 40-44) which corresponds well to the calculated $M_r$ and pI of the protein, and another group (numbered 28, 228) which has a substantially lower $M_r$ than expected suggesting that these proteins may represent an isoform(s) or a fragment(s) of the enzyme.

| Spotnr | Protein name | *NCBI* | Direct analysis mascot score | | Ziptip mascot score | | Mass | pI |
|---|---|---|---|---|---|---|---|---|
| | | | PMF | MS/MS | PMF | MS/MS | | |
| 1 | Peptidylprolyl isomerase A (cyclophilin A) | 8394009 | 86 | 223 | | | 18171 | 8.34 |
| 2 | Peptidylprolyl isomerase A (cyclophilin A) | 8394009 | 57 | 67 | | | 18171 | 8.34 |
| 3 | Peptidylprolyl isomerase A (cyclophilin A) | 8394009 | 74 | | | | 18171 | 8.34 |
| 4 | RIKEN cDNA 0810011J09 (similar to ubiquitin-conj-enzyme E2) | 20341854 | | | 96 | | 16570 | 7.74 |
| 5 | | | | | | | | |
| 6 | Sorting nexin 3 | 4507143 | | | 118 | | 18840 | 8.71 |
| 7 | Cofilin 1, non-muscle | 8393101 | 77 | 130 | | | 18813 | 8.22 |
| 8 | Cofilin 1, non-muscle | 8393101 | | | 69 | | 18813 | 8.22 |
| 9 | Similar to SMT3 suppressor of mif two 3 homolog 2 (yeast) | 18564783 | 65 | | | | 15012 | 10.03 |
| | ISS-homolog to hypothetical 18.5 kDa protein | 12837887 | 38 | 137 | | | 21046 | 9.46 |
| 10 | *Superoxide dismutase 2* | 8394331 | 71 | 173 | | | 24919 | 8.96 |
| 11 | Peroxiredoxin 1 | 16923958 | 76 | 43 | 102 | | 22371 | 8.27 |
| | *Superoxide dismutase 2* | 8394331 | 52 | 306 | 76 | | 24919 | 8.96 |
| | Similar to TOB3 | 20901979 | | | 41 | | 21691 | 9.27 |
| | Enhancer protein | 2135068 | | | 69 | | 22503 | 8.39 |
| 12 | Adenylate kinase 1 | 13242235 | 120 | 266 | | | 21741 | 7.71 |
| 13 | Neuronal protein 22 | 18252579 | 81 | 172 | | | 22817 | 6.84 |
| 14 | *Superoxide dismutase 2* | 8394331 | 62 | | 74 | | 24919 | 8.96 |
| | Cystaine-rich protein | 8393206 | 38 | | 73 | | 21503 | 8.90 |
| | Similar to TOB3 | 20901979 | | | 43 | | 21691 | 9.27 |
| 15 | *Superoxide dismutase 2* | 8394331 | 50 | 252 | 77 | | 24919 | 8.96 |
| 16 | *Superoxide dismutase 2* | 8394331 | | | 37 | | 24919 | 8.96 |
| 17 | Hypothetical protein XP088406 | 18601785 | | 55 | | | 22020 | 8.10 |
| 18 | Similar to brain-specific protein p25 $\alpha$ | 20909043 | 98 | 127 | | | 23763 | 9.46 |
| 19 | *Glutamate oxaloacetate transaminase 2* | 6980972 | 50 | 111 | 41 | 85 | 47875 | 9.13 |
| | Brain 3-Hydroxyacyl-Coa Dehydrogenase | 14488727 | | | 48 | 9 | 27276 | 8.91 |
| 20 | *Voltage-dependent anion channel 1* | 6755963 | | | 70 | 171 | 30883 | 8.62 |
| 21 | Triosephosphate isomerase 1 | 12621074 | | | 94 | | 27465 | 6.45 |
| 22 | Triosephosphate isomerase 1 | 12621074 | 42 | | 121 | 182 | 27465 | 6.45 |
| 23 | Phosphoglycerate mutase type B subunit | 12844989 | | | 142 | | 29038 | 7.07 |
| 24 | Triosephosphate isomerase 1 | 12621074 | 42 | | | | 27465 | 6.45 |
| 25 | Heat shock protein J2 | 19855061 | | | 107 | 40 | 26993 | 6.99 |
| 26 | 4-Nitrophenylphosphatase domain and non-neuronal SNAP25-like protein | 6679066 | | | 59 | | 33682 | 9.48 |
| 27 | Similar to AU RNA-binding protein/enoyl-Coenzyme A hydratase | 20072952 | 126 | 200 | | | 33002 | 9.57 |
| 28 | Glyceraldehyde-3-phosphate dehydrogenase | 8393418 | 64 | 107 | | | 36250 | 8.14 |
| 29 | RIKEN cDNA 1200007021 | 20820786 | 112 | 190 | | | 31652 | 8.54 |
| | Unnamed protein product | 21756059 | 107 | | | | 27198 | 8.48 |
| 30 | *Voltage-dependent anion channel 1* | 6755963 | 86 | 308 | | | 30883 | 8.62 |
| 31 | *Voltage-dependent anion channel 1* | 6755963 | 103 | 245 | | | 30883 | 8.62 |
| 32 | V-ATPase E2 subunit | 20799123 | 90 | 41 | | | 26388 | 8.44 |
| 33 | *Voltage-dependent anion channel 1* | 6755963 | 58 | 115 | | | 30883 | 8.62 |
| 34 | | | | | | | | |
| 35 | *Voltage-dependent anion channel 1* | 6755963 | 58 | 13 | 136 | 462 | 30883 | 8.62 |
| 36 | *Voltage-dependent anion channel 2* | 13786202 | 98 | 135 | | | 32385 | 7.44 |
| 37 | *Voltage-dependent anion channel 2* | 13786202 | | 98 | 113 | | 32385 | 7.44 |
| 38 | Similar to Myeloid leukemia factor 2 | 20832291 | 73 | 84 | | | 28389 | 6.40 |
| | Hypothetical protein XP_166785 | 20559014 | 44 | | | | 31499 | 6.82 |
| 39 | *Malate dehydrogenase (EC 1,1,1,37) precursor* | 319830 | 166 | 436 | | | 36157 | 8.93 |
| 40 | Glyceraldehyde-3-phosphate dehydrogenase | 8393418 | 92 | 145 | | | 38250 | 8.14 |
| 41 | Glyceraldehyde-3-phosphate dehydrogenase | 8393418 | 125 | 153 | | | 36250 | 8.14 |
| 42 | Glyceraldehyde-3-phosphate dehydrogenase | 8393418 | 170 | | | | 36250 | 8.14 |
| 43 | Glyceraldehyde-3-phosphate dehydrogenase | 8393418 | 156 | 112 | | | 36250 | 8.14 |
| 44 | Glyceraldehyde-3-phosphate dehydrogenase | 8393418 | 54 | 65 | | | 36250 | 8.14 |
| 45 | | | | | | | | |
| 46 | *Voltage-dependent anion channel 2* | 6093769 | | | 73 | | 32385 | 7.44 |
| 47 | LIM and SH3 protein 1 | 14249130 | 148 | 226 | 165 | | 30462 | 6.61 |

TABLE I—*continued*

| Spotnr | Protein name | *NCBI* | Direct analysis mascot score | | Ziptip mascot score | | Mass | pI |
|---|---|---|---|---|---|---|---|---|
| | | | PMF | MS/MS | PMF | MS/MS | | |
| 48 | *Glutamate oxaloacetate transaminase 2* | 6980972 | 168 | 248 | | | 47875 | 9.13 |
| 49 | *Glutamate oxaloacetate transaminase 2* | 8980972 | 187 | 341 | | | 47875 | 9.13 |
| 50 | *Glutamate oxaloacetate transaminase 2* | 6980972 | 142 | 171 | | | 47875 | 9.13 |
| 51 | Aldolase A, fructose-bisphosphate | 6978487 | 269 | 488 | | | 39847 | 8.31 |
| 52 | Aldolase A, fructose-bisphosphate | 6978487 | 239 | 338 | | | 39847 | 8.31 |
| 53 | Aldolase A, fructose-bisphosphate | 6978487 | 286 | 343 | | | 39847 | 8.31 |
| 54 | Aldolase A, fructose-bisphosphate | 6978487 | 178 | 268 | | | 39847 | 8.31 |
| 55 | Aldolase C, fructose-bisphosphate | 6978489 | 167 | 180 | | | 39738 | 6.67 |
| 56 | Aldolase C, fructose-bisphosphate | 6978489 | 46 | | | | 39738 | 6.67 |
| | Poly(rC)-binding protein 1 | 6754994 | 45 | 52 | | | 38179 | 6.66 |
| 57 | Aldo-keto reductase family 1, member A1 | 13591894 | 45 | | | | 36791 | 6.84 |
| 58 | Succinate-CoA ligase | 16758586 | | | 72 | | 35521 | 9.54 |
| | SW1/SNF-related, matrix-associated, actin-dependent regulator of chromatin | 3687440 | | | 67 | | 37934 | 9.02 |
| 59 | *Creatine kinase, ubiquitous mitochondrial* | 125316 | | 12 | | | 47542 | 8.72 |
| 60 | *Creatine kinase, ubiquitous mitochondrial* | 125316 | 54 | 76 | | | 47542 | 8.72 |
| 61 | *Creatine kinase, ubiquitous mitochondrial* | 125316 | 91 | 83 | | | 47542 | 8.72 |
| 62 | *Creatine kinase, ubiquitous mitochondrial* | 125316 | 88 | 114 | | | 47542 | 8.72 |
| 63 | *Glutamine synthetase (glutamate-ammonia ligase)* | 8393456 | 158 | 209 | | | 43209 | 6.93 |
| 64 | *Glutamine synthetase (glutamate-ammonia ligase)* | 8393456 | 158 | | | | 43209 | 6.93 |
| 65 | *Similar to elongation factor Tu* | 20864488 | 72 | 131 | 173 | | 50042 | 7.65 |
| 66 | Poly(rC)-binding protein 1; poly(rC)-binding protein 1 | 6754994 | 39 | 22 | | | 38179 | 6.66 |
| 67 | Poly(rC)-binding protein 1; poly(rC)-binding protein 1 | 6754994 | 27 | | | | 38179 | 6.66 |
| 68 | Purine rich element-binding protein A; Pur alpha | 6679573 | 78 | 44 | | | 35056 | 6.07 |
| 69 | CDCrel-1Al | 19909845 | 129 | 127 | | | 42615 | 6.21 |
| 70 | Peanut (Drosophila)-like 1 | 16758814 | 185 | 91 | | | 44540 | 6.34 |
| 71 | Synapsin II | 9507161 | 49 | 141 | | | 53076 | 8.05 |
| 72 | Synapsin II | 9507161 | 95 | 214 | | | 53076 | 8.05 |
| 73 | Synapsin II | 9507161 | 120 | 116 | | | 53076 | 8.05 |
| 74 | Synapsin II | 9507161 | 87 | 22 | | | 53076 | 8.05 |
| | Fibrinogen B-$\beta$-chain | 529586 | 54 | | | | 34574 | 8.06 |
| 75 | Synapsin II | 9507161 | 92 | 300 | | | 53076 | 8.05 |
| 76 | Glutamate dehydrogenase | 6980956 | 124 | 85 | | | 61943 | 8.05 |
| | $Ca^{2+}$/calmodulin-dependent protein kinase II $\alpha$ | 6978593 | 35 | | | | 54779 | 6.61 |
| 77 | $Ca^{2+}$/calmodulin-dependent protein kinase II $\alpha$ | 6978593 | 36 | | | | 54779 | 6.61 |
| 78 | Synapsin II | 9507161 | 60 | 137 | | | 53076 | 8.05 |
| 79 | Hypothetical protein FLJ10849 | 21619404 | 61 | | 147 | | 49787 | 6.36 |
| | $Ca^{2+}$/calmodulin-dependent protein kinase II $\alpha$ | 6978593 | 57 | 23 | 104 | | 54779 | 6.61 |
| | IP3K | 13591973 | | | 44 | | 50548 | 7.64 |
| 80 | Hypothetical protein FLJ10849 similar to septin 6 | 21619404 | 135 | 44 | 166 | | 49787 | 6.36 |
| | $Ca^{2+}$/calmodulin-dependent protein kinase II $\alpha$ | 6978593 | 41 | 11 | 70 | | 54779 | 6.61 |
| | IP3K | 13591973 | | | 59 | | 50548 | 7.64 |
| 81 | Similar to actin-related protein 2/3 | 13569956 | | 14 | | | 16979 | 6.15 |
| 82 | $\kappa$ B-ras 1 | 9966809 | 69 | 8 | | | 21864 | 6.00 |
| 83 | *Voltage-dependent anion channel 2* | 13786202 | 69 | | | | 32385 | 7.44 |
| 84 | Similar to myeloid leukemia factor 2 | 20832291 | | | 65 | 28 | 28398 | 6.40 |
| 85 | Tropomyosin 1 $\alpha$, brain | 112440 | | | 70 | | 32613 | 4.72 |
| 86 | | | | | | | | |
| 87 | Glutathione transferase $\omega$1 | 12585231 | | 16 | 95 | | 28064 | 6.25 |
| 88 | | | | | | | | |
| 89 | Calmodulin 2 (phosphorylase kinase, $\delta$) | 4502549 | 53 | 94 | | | 16987 | 4.09 |
| 90 | Ubiquitin carboxyl-terminal hydrolase L1 | 8394506 | | | 55 | | 25204 | 5.12 |
| 91 | | | | | | | | |
| 92 | Similar to $\alpha$-tubulin [Mus musculus] | 20884391 | 56 | 50 | | | 19053 | 5.46 |
| 93 | Similar to $\alpha$-tubulin | 20884391 | 49 | 45 | 51 | | 19053 | 5.46 |
| 94 | ISS-homolog to UMP-CMP KINASE (EO 2.7.1.48)-putative | 12836302 | | | 78 | | 22459 | 5.68 |
| 95 | | | | | | | | |
| 96 | Similar to initiation factor 5A (elF-5A) (elF-4D) | 20881893 | | | 54 | 35 | 14218 | 5.51 |
| 97 | *ATP synthase subunit d* | 9508411 | | | 81 | 48 | 18873 | 6.17 |
| 98 | Similar to BAG-family molecular chaperone regulator-2 | 20845162 | 36 | | 117 | | 23726 | 6.01 |
| 99 | Tumor protein D52-like 1; hD53 | 4507641 | 45 | | | | 22620 | 5.46 |
| 100 | F-actin capping protein $\beta$ subunit; Cap Z | 4826659 | 76 | 38 | | | 31063 | 5.69 |
| 101 | F-actin capping protein $\beta$ subunit; Cap Z | 4826659 | 102 | | | | 31063 | 5.69 |
| | *Prohibitin* | 6679299 | 90 | | | | 29875 | 5.57 |
| 102 | STIP1 homology and U-box-containing protein 1 | 9789907 | | | 43 | | 35455 | 5.71 |
| 103 | Capping protein $\alpha$ 2 | 6671672 | 63 | 3 | 175 | 21 | 33166 | 5.57 |
| 104 | SNAP25 interacting protein 30 | 22129759 | 113 | 85 | 139 | 11 | 30359 | 5.88 |
| 105 | Tubulin $\beta$ chain 15 | 92930 | 86 | 16 | 140 | | 50665 | 4.79 |
| | Protein phosphatase 1, catalytic subunit, $\alpha$ isoform | 13994195 | | | 70 | | 38353 | 5.94 |
| 106 | Acidic ribosomal protein PO | 11693176 | 33 | | 72 | | 34509 | 5.91 |
| 107 | *Serine racemase* | 7305521 | 46 | | | | 36799 | 5.68 |
| 108 | Tubulin $\beta$ chain 15 | 92930 | 125 | 21 | | | 50665 | 4.79 |
| 109 | SNAP25 interacting protein 30 | 22129759 | 59 | | 97 | | 30359 | 5.88 |
| | LIM and SH3 protein 1 | 14249130 | | | 45 | | 30462 | 6.61 |
| 110 | Vesl-1L | 13928988 | 117 | | | | 41422 | 5.39 |
| 111 | Regulator of G-protein signaling 19 interacting protein 1 | 9055336 | 29 | | 134 | | 36349 | 5.65 |
| 112 | Annexin V | 999934 | | | 43 | | 35879 | 5.03 |

TABLE I—*continued*

| Spotnr | Protein name | *NCBI* | Direct analysis mascot score | | Ziptip mascot score | | Mass | pI |
|---|---|---|---|---|---|---|---|---|
| | | | PMF | MS/MS | PMF | MS/MS | | |
| 113 | Purine rich element binding protein A; Pur α | 6679573 | 89 | | 128 | | 35056 | 6.07 |
| 114 | CDCrel-1A1 | 19909845 | 118 | | 175 | | 42615 | 6.21 |
| 115 | CDCrel-1A1 | 19909845 | 94 | | | | 42615 | 6.21 |
| 116 | Tubulin β chain 15 | 92930 | 99 | 8 | 103 | | 50665 | 4.79 |
| 117 | Tubulin β chain 15 | 92930 | 83 | 58 | | | 50665 | 4.79 |
| 118 | STIP1 homology; carboxyl terminus of Hsp70-interacting protein | 9789907 | | | 70 | | 35455 | 5.71 |
| 119 | G protein β 2 subunit | 1730213 | | 11 | 85 | | 38295 | 5.47 |
| 120 | Tubulin β chain 15 | 92930 | 108 | 45 | | | 50665 | 4.79 |
| 121 | Tubulin α | 223556 | 82 | 40 | | | 51054 | 4.94 |
| 122 | Tubulin α | 223556 | 56 | 38 | | | 51054 | 4.94 |
| 123 | Tubulin α | 223556 | 66 | 46 | | | 51054 | 4.94 |
| 124 | Actin β | 71620 | 105 | 138 | 125 | | 42338 | 5.29 |
| 125 | Succinyl-CoA ligase | 21263999 | 106 | 27 | | | 46765 | 5.65 |
| 126 | Actin β | 71620 | 82 | | | | 42338 | 5.29 |
| 127 | Tubulin β chain 15 | 92930 | | | 55 | | 50665 | 4.79 |
| 128 | Creatine kinase, brain | 6978659 | 63 | 22 | | | 43160 | 5.33 |
| 129 | Vesl-1L | 13928988 | 191 | 90 | | | 41422 | 5.39 |
| 130 | Neurofilament triplet M protein | 128149 | 111 | 16 | | | 52017 | 5.15 |
| | Creatine kinase, brain | 6978659 | 33 | 41 | | | 43160 | 5.33 |
| 131 | Vesl-1L | 13928988 | 234 | 140 | | | 41422 | 5.39 |
| 132 | Actin β | 71620 | 63 | 136 | | | 42338 | 5.29 |
| 133 | Actin β | 71620 | 117 | 217 | | | 42338 | 5.29 |
| 134 | Neurofilament triplet M protein | 128149 | 109 | | | | 52017 | 5.15 |
| 135 | Vesl-1L | 3452560 | 128 | 81 | | | 41485 | 5.46 |
| 136 | Actin β | 71620 | 116 | 379 | | | 42338 | 5.29 |
| 137 | Actin β | 71620 | 161 | 384 | | | 42338 | 5.29 |
| 138 | Actin β | 71620 | 150 | 365 | | | 42338 | 5.29 |
| 139 | Actin β | 71620 | 161 | 379 | | | 42338 | 5.29 |
| 140 | Neurofilament, light polypeptide | 13929098 | 95 | 56 | | | 61531 | 4.63 |
| | Actin β | 71620 | 62 | 178 | | | 42338 | 5.29 |
| 141 | e-Tropomodulin | 6981658 | 99 | 21 | | | 40770 | 4.97 |
| | Actin β | 71620 | 99 | 126 | | | 42338 | 5.29 |
| 142 | Neurofilament, light polypeptide | 13929098 | 41 | 7 | | | 61531 | 4.63 |
| | Vesl-1L | 3452560 | | 40 | | | 41485 | 5.46 |
| 143 | *Glial fibrillary acidic protein delta* | 5030428 | 206 | 45 | | | 48953 | 5.72 |
| | Vesl-1L | 3452560 | 104 | 122 | | | 41485 | 5.46 |
| 144 | Tubulin β chain 15 | 92930 | 147 | 162 | | | 50665 | 4.79 |
| 145 | Tubulin β chain 15 | 92930 | 214 | 189 | | | 50665 | 4.79 |
| 146 | Neurofilament, light polypeptide | 13929098 | 293 | 160 | | | 61531 | 4.63 |
| 147 | Neurofilament, light polypeptide | 13929098 | 75 | 34 | | | 61531 | 4.63 |
| 148 | Neurofilament, light polypeptide | 13929098 | 293 | | | | 61531 | 4.63 |
| | Proteasome (prosome, macropain) 26S subunit, ATPase 9 | 13928808 | | | 116 | | 50078 | 5.04 |
| | Protein kinase C and casein kinase substrate in neurons 1 (Syndapin) | 8393896 | | | 107 | | 50952 | 5.15 |
| | Tubulin β chain 15 | 92930 | | | 68 | | 50865 | 4.79 |
| | *Glial fibrillary acidic protein* | 8393431 | | | 67 | | 50146 | 5.35 |
| 149 | Neurofilament, light polypeptide | 13929098 | 155 | 39 | | | 61531 | 4.63 |
| 150 | Tubulin β chain 15 | 92930 | 118 | 46 | | | 50665 | 4.79 |
| 151 | *Glial fibrillary acidic protein δ* | 5030428 | 261 | 123 | | | 48953 | 5.72 |
| | Neurofilament, light polypeptide | 13929098 | 98 | 52 | | | 61531 | 4.63 |
| 152 | Tubulin β chain 15 | 92930 | 107 | 132 | | | 50665 | 4.79 |
| 153 | Tubulin α | 223556 | 103 | 215 | | | 51054 | 4.94 |
| 154 | *Glial fibrillary acidic protein* | 8393431 | 393 | 299 | | | 50146 | 5.35 |
| 155 | Tubulin α | 223556 | 109 | 215 | | | 51054 | 4.94 |
| 156 | Tubulin α | 223556 | 142 | 367 | | | 51054 | 4.94 |
| 157 | Tubulin α | 223556 | 82 | 348 | | | 51054 | 4.94 |
| 158 | Tubulin β chain 15 | 92930 | 148 | 135 | 189 | | 50865 | 4.79 |
| 159 | Tubulin α | 223556 | 69 | 126 | | | 51054 | 4.94 |
| 160 | Neurofilament, light polypeptide | 13929098 | 294 | 158 | | | 61531 | 4.63 |
| 161 | Internexin, α | 9506811 | 260 | 146 | | | 56317 | 5.20 |
| 162 | Tubulin α | 223556 | | 7 | 71 | | 51054 | 4.94 |
| | Internexin, α | 55622 | | | 81 | | 55776 | 5.20 |
| 163 | Tubulin α | 223556 | 53 | 19 | | | 51054 | 4.94 |
| 164 | Internexin, α | 9506811 | 39 | | | | 56317 | 5.20 |
| 165 | Internexin, α | 9506811 | 280 | 80 | 287 | | 56317 | 5.20 |
| 166 | Internexin, α | 9506811 | 297 | 310 | | | 56317 | 5.20 |
| 167 | Internexin, α | 9506811 | 280 | | | | 56317 | 5.20 |
| 168 | Internexin, α | 9506811 | 408 | 259 | | | 56317 | 5.20 |
| 169 | Internexin, α | 9506811 | 360 | 363 | | | 56317 | 5.20 |
| 170 | Internexin, α | 9506811 | 366 | 201 | | | 56317 | 5.20 |
| 171 | | | | | | | | |
| 172 | Hypothetical protein similar to adenylate kinase 5 | 13874609 | | | 66 | | 49572 | 4.99 |
| 173 | Heat shock 70kD protein 8; Heat shock cognate protein 70 | 13242237 | 145 | 107 | | | 71263 | 5.37 |
| | Neurofilament, light polypeptide | 13929098 | 64 | 13 | | | 61531 | 4.63 |
| 174 | Heat shock 70kD protein 8; Heat shock cognate protein 70 | 13242237 | | | 233 | | 71263 | 5.37 |
| | Neurofilament, light polypeptide | 13929098 | | | 137 | | 61531 | 4.63 |
| 175 | Heat shock 70kD protein 8; Heat shock cognate protein 70 | 13242237 | 249 | 407 | | | 71263 | 5.37 |
| 176 | Heat shock 70kD protein 8; Heat shock cognate protein 70 | 13242237 | 109 | 26 | | | 71263 | 5.37 |

TABLE I—*continued*

| Spotnr | Protein name | *NCBI* | Direct analysis mascot score | | Ziptip mascot score | | Mass | pI |
|---|---|---|---|---|---|---|---|---|
| | | | PMF | MS/MS | PMF | MS/MS | | |
| 177 | Neurofilament protein, middle polypeptide | 8393823 | 64 | 90 | | | 95885 | 4.76 |
| 178 | Synapse-associated protein SAP90 | 9665227 | 136 | | 174 | | 80902 | 5.58 |
| 179 | Synapse-associated protein SAP90 | 9665227 | 181 | 244 | 330 | | 80902 | 5.58 |
| 180 | Synapse-associated protein SAP90 | 9665227 | 227 | 99 | 276 | | 80902 | 5.58 |
| 181 | Internexin $\alpha$ | 9506811 | 202 | 97 | | | 56317 | 5.20 |
| 182 | Tubulin $\beta$ chain 15 | 92930 | | | 130 | | 50665 | 4.79 |
| 183 | Tubulin $\beta$ chain 15 | 92930 | 110 | 106 | 176 | | 50665 | 4.79 |
| 184 | *Vimentin* | 14389299 | 48 | | | | 53933 | 5.06 |
| | Tubulin $\beta$ chain 15 | 92930 | 48 | 11 | | | 50665 | 4.79 |
| 185 | Internexin, $\alpha$ | 9506811 | 216 | 72 | | | 56317 | 5.20 |
| 186 | *Vimentin* | 14389299 | 41 | | | | 53933 | 5.06 |
| 187 | Actin-like 6 | 13937393 | | | 75 | 41 | 47573 | 5.48 |
| 188 | | | | | | | | |
| 189 | Tubulin $\alpha$ | 223556 | | | 50 | | 51054 | 4.94 |
| 190 | Heterogeneous nuclear ribonucleoprotein H | 9845253 | 54 | 14 | | | 49821 | 5.89 |
| 191 | Heterogeneous nuclear ribonucleoprotein H | 9845253 | 37 | | 64 | | 49821 | 5.89 |
| 192 | Heterogeneous nuclear ribonucleoprotein H | 10946928 | 48 | 93 | | | 49693 | 5.89 |
| 193 | Heterogeneous nuclear ribonucleoprotein H | 9845253 | 90 | 122 | | | 49821 | 5.89 |
| 194 | Tubulin $\beta$ chain 15 | 92930 | 68 | 44 | | | 50665 | 4.79 |
| 195 | Tubulin $\beta$ chain 15 | 92930 | 97 | 74 | | | 50665 | 4.79 |
| 196 | Tubulin $\alpha$ | 223556 | 76 | 247 | | | 51054 | 4.94 |
| 197 | | | | | | | | |
| 198 | | | | | | | | |
| 199 | Unknown protein for MGC | 17391508 | | 20 | | | 73624 | 5.96 |
| 200 | Chaperonin subunit 2 ($\beta$) | 57993 | 62 | | | | 57993 | 5.97 |
| | Tubulin $\beta$ chain 15 | 92930 | 63 | 29 | | | 50665 | 4.79 |
| 201 | Septin 6 | 14714696 | 56 | 24 | | | 49324 | 6.12 |
| 202 | Tubulin $\alpha$ | 223556 | 50 | | | | 51054 | 4.94 |
| | Similar to Putative serine/threonine-protein kinase P78 | 20910752 | | 20 | | | 53170 | 5.80 |
| 203 | | | | | | | | |
| 204 | Synapsin II | 6885997 | 101 | 268 | | | 63974 | 8.73 |
| 205 | Synapsin II | 6685997 | 104 | 96 | | | 63974 | 8.73 |
| 206 | Synapsin II | 6685997 | 90 | 307 | | | 63974 | 8.73 |
| 207 | Synapsin II | 6685997 | 107 | 186 | | | 63974 | 8.73 |
| 208 | Synapsin II | 6685997 | 90 | 285 | | | 63974 | 8.73 |
| 209 | Synapsin II | 6685997 | 140 | 188 | | | 63974 | 8.73 |
| 210 | Synapsin Ib | 112348 | 93 | 68 | | | 70038 | 9.84 |
| 211 | Brain-specific angiogenesis inhibitor 1-associated protein 2 (IRSP 53) | 17105348 | 202 | | | | 57974 | 8.97 |
| | Synapsin Ib | 112348 | 153 | 320 | | | 70038 | 9.84 |
| 212 | Synapsin Ib | 112348 | 116 | 435 | | | 70038 | 9.84 |
| 213 | Synapsin I | 9507159 | 71 | 100 | | | 74354 | 9.81 |
| 214 | Synapsin I | 9507159 | 50 | 49 | | | 74354 | 9.81 |
| 215 | Synapsin Ib | 112348 | 62 | 75 | | | 70038 | 9.84 |
| 216 | Similar to DnaJ-like protein | 20830104 | 53 | 60 | | | 44949 | 6.65 |
| 217 | | | | | | | | |
| 218 | Translationally controlled tumor protein TCTP | 20876984 | 40 | 147 | | | 19552 | 4.93 |
| 219 | Similar to Tubulin $\beta$-2 chain | 20822920 | 28 | 17 | | | 23872 | 5.49 |
| 220 | | | | | | | | |
| 221 | Ubiquitin carboxyl-terminal hydrolase L1 | 8394506 | 14 | | | | 25204 | 5.12 |
| 222 | Synapse-associated protein SAP90 | 9665227 | 157 | 150 | | | 80902 | 5.58 |
| | PSD-95-binding protein | 7514059 | | 36 | | | 76944 | 5.56 |
| 223 | *DnaK-type molecular chaperone grp75 precursor* | 2119726 | 81 | 138 | | | 73984 | 5.87 |
| 224 | Heat shock 70 kDa protein 1 | 2506541 | 62 | 55 | | | 70236 | 5.46 |
| 225 | | | | | | | | |
| 226 | Synapsin II | 6685997 | 66 | 275 | | | 63974 | 8.73 |
| 227 | Synapsin II | 6685997 | 76 | 287 | | | 63974 | 8.73 |
| 228 | Glyceraldehyde-3-phosphate dehydrogenase | 8393418 | | 47 | | | 36250 | 8.14 |
| 229 | Spinophilin: neurabin II | 16758228 | 91 | 28 | | | 89706 | 4.86 |
| | Rabaptin 5 | 9506845 | 65 | 27 | | | 99366 | 4.95 |
| 230 | $\alpha$ II spectrin | 1495198 | 275 | 128 | | | 285218 | 5.19 |
| 231 | $\alpha$ II spectrin | 1495198 | 327 | 218 | | | 285218 | 5.19 |
| 232 | $\alpha$ II spectrin | 1495198 | 311 | 17 | | | 285218 | 5.19 |
| 233 | Neurofilament triplet M protein | 482393 | 38 | 34 | | | 95719 | 4.78 |
| 234 | Eukaryotic translation elongation factor 1 $\alpha$ 2 | 15805031 | 74 | 66 | | | 50652 | 9.10 |
| 235 | *Malate dehydrogenase (EC 1.1.1.37) precursor* | 319830 | 39 | | | | 36157 | 8.93 |
| 236 | Synapsin Ib | 112348 | 111 | 161 | | | 70038 | 9.84 |
| 237 | Synapsin Ib | 112348 | 61 | 42 | | | 70038 | 9.84 |
| 238 | *Mitochondrial aconitase* | 13242312 | 74 | 54 | | | 86354 | 7.87 |
| 239 | | | | | | | | |
| 240 | Alpha II spectrin | 1495198 | 99 | | | | 285218 | 5.19 |
| 241 | Eukaryotic translation initiation factor 4E | 6681293 | | 10 | | | 25037 | 5.79 |
| 242 | | | | | | | | |
| 243 | $\gamma$ tubulin expressed seq. | 19527242 | | 20 | | | 51069 | 5.66 |
| 244 | F-actin capping protein $\beta$ subunit; Cap Z | 4826659 | 28 | 18 | | | 31063 | 5.69 |
| 245 | 78 KDa glucose-regulated protein grp 78 | 121574 | 85 | 101 | | | 72633 | 5.07 |
| 246 | Synapse-associated protein SAP90 | 9665227 | 71 | 47 | | | 80758 | 5.58 |
| 247 | Synapse-associated protein SAP90 | 9665227 | 102 | 95 | | | 80758 | 5.58 |

TABLE I—*continued*

| Spotnr | Protein name | *NCBI* | Direct analysis mascot score | | Ziptip mascot score | | Mass | pI |
|---|---|---|---|---|---|---|---|---|
| | | | PMF | MS/MS | PMF | MS/MS | | |
| 248 | Synapsin Ib | 112348 | 52 | 71 | | | 70038 | 9.84 |
| 249 | Brain-specific angiogenesis inhibitor 1-associated Protein 2 (IRSP 53) | 17105348 | 67 | | | | 57974 | 8.97 |
| | Calmodulin-dependent protein kinase II $\alpha$ | 6978593 | 48 | 112 | | | 54081 | 6.61 |
| 250 | Brain-specific angiogenesis inhibitor 1-associated protein 2 (IRSP 53) | 17105348 | 74 | | | | 57974 | 8.97 |
| | Calmodulin-dependent protein kinase II $\alpha$ | 6978593 | 35 | 76 | | | 54081 | 6.61 |

study some major PSD proteins are under-represented, for example, glutamate receptor subunits are not detected although they are known to be highly enriched in the PSD fraction. To address this problem we performed an independent experiment, using the newly developed method that combines the ICAT derivatization/purification protocol of the trypsin-digested PSD to nanoliquid chromatography-electrospray tandem mass spectrometry (27–29).

Briefly, the purified sample is labeled with the ICAT® reagent and then digested with trypsin to produce tryptic peptides. The ICAT® reagent consists of three basic moieties; a biotin tag, a variable linker region ($^{12}$C or $^{13}$C-labeled that is used if two similar samples are to be analyzed to discover their relative differences in terms of protein expression, one labeled with the $^{12}$C version the other with the $^{13}$C) and a cysteine reactive portion. Thus, derivatization with the ICAT® reagent generates cysteine-containing peptides that can be affinity selected from non-derivatized peptides using avidin chromatography, substantially reducing sample complexity. These derivatized peptides can then be analyzed by LC-MS/MS, producing daughter ion spectra by collision-induced dissociation that can be analyzed by data base searching to reveal the identity of the protein from which they were digested. If two similar samples are to be analyzed (*e.g.* control and treated) relative changes in protein expression can be inferred from relative differences in intensity of the $^{12}$C and $^{13}$C-labeled (but otherwise identical) peptides. However, the purpose of experiments described here was identification rather than relative quantitation.

Thus, after elution from the avidin affinity column, the cysteine-containing peptides were resolved by reversed phase liquid chromatography and sprayed online into an electrospray tandem mass spectrometer. Peptides were selected automatically in a data-dependent manner and fragmented in a collision cell. Two independent experiments have been performed. In the first ICAT experiment a total of about 1200 MS/MS spectra were generated. The spectra were searched against the Pro ICAT™ engine. Approximately 850 MS/MS spectra gave protein hits, of which 356 of the putatively identified proteins have a score of 10 or above. In the second ICAT experiment 202 putatively identified proteins have a score of at least 10. While the two-dimensional gel electrophoresis type experiment has several criteria to assist the assignment of the protein identifications, such as PMF, MS/MS data, pI and $M_r$ of the protein spots, LC-MS/MS experiments rely solely on the MS/MS spectra for the protein identification, and therefore may be prone to the generation of false positive results. To filter off false positive results we firstly excluded any protein identifications other than those from rat. The data from the two ICAT experiments were pooled together for the analysis. When two distinct peptides from different MS/MS spectra with scores above 10 can be matched to a single protein, it is considered as significant. For proteins that are matched by single peptides a more stringent criterion is applied; the confidence must be at least 70 and the score at least 20. In total 61 proteins are identified after the application of these stringent conditions (Table III). We believe that we have excluded all the false positive results. Furthermore, the identified proteins, especially those detected with multiple peptides, are most likely the abundant proteins of the PSD fraction. Proteins of lower abundance generally gave lower quality mass spectra or may be detected but not selected for MS/MS analysis. Therefore, it is inevitable that there are a number of false negative results. For example, proteins that are known or expected to be present in the PSD such as dynamin, parkin, 14-3-3 protein, cadherin, Epac, and MAGI-3 etc., are detected at lower confidence and/or score and are not listed in Table III. Thus, in summary Table III represents proteins that have been confidently identified using an ICAT LC-MS/MS approach.

In addition to the cytosolic proteins and cytoskeletal proteins, the LC-MS/MS experiment allows the detection of membrane proteins such as the ionotropic glutamate receptors and ion channels, large proteins (plectin > 500 kDa) and basic proteins (ribosomal protein > pH 10) that were not detected by the two-dimensional gel approach (Table II). Some of the proteins identified here have been previously reported to be present in PSD, *e.g.* band 83 (PMES-; Ref. 30) and Esau, but their functions are currently unknown and are not listed in Table II. In short, the ICAT LC-MS/MS and the two-dimensional gel MS and MS/MS experiments are complementary.

## DISCUSSION

The PSD is considered to be a tightly packed protein complex containing receptors and ion channels that are anchored and clustered by scaffold proteins. There are a number of signaling proteins that regulate the intrinsic properties of the receptors, and that may modulate their trafficking via the actions of the cytoskeletal proteins (9–10).

Our proteome study indicates that the PSD has a high protein complexity and also indicates novel aspects of functionality. Broadly, we can distinguish two salient features of the PSD proteins. First, two-dimensional gel experiments reveal that many distinct proteins are detected as multiple protein spots, suggesting that they exist as isoforms or are post-translationally modified. Phosphorylation and to a lesser extent palmitoylation and nitrosylation of a number of PSD proteins have been implicated in the regulation of the proteins' intrinsic properties and/or their trafficking and localization, and play important roles in the induction and expression of synaptic plasticity (31–33). Second, the PSD contains distinct functional groups of proteins that might be involved in diverse physiological activities of the PSD. The groups include proteins of the cytoskeleton and their interaction partners for the maintenance and modulation of synaptic architecture; proteins involved in sorting and trafficking of membrane proteins; components for the anaerobic energy supply for the acute requirement of energy during intense neuronal activity; the proteasome system for specific synaptic protein degradation; the chaperone system for correct protein folding; the local protein synthetic machinery; the scaffold and signaling protein complexes and of course the receptors, ion channels and adhesion molecules. There are also a number of proteins with unknown function, and proteins that currently are not known to fall into one of the above-mentioned functional categories. The interpretation of their physiological significance in PSD functioning is not clear at the moment, and

therefore these proteins are not further discussed in the present study.

*The Scaffold and Adaptor Proteins*—The scaffold and adaptor proteins in the PSD regulate the assembly and function of the complex macromolecular protein network. They cluster diverse receptors/ion channels, signaling proteins and adhesion molecules in the PSD, and link them further to the cytoskeletal proteins (9–11, 33–34). In accordance to previous studies the immunoblot experiment reveals the enrichment of SAP90/PSD-95 in PSD. Interestingly, the two-dimensional gel displays 6 forms of SAP90/PSD-95, 6 forms of vesl-1L/homer and a single PSD-95-binding protein (GKAP/SAPAP1). The chemical diversity of these single proteins suggests the existence of multiple post-translationally modified forms. Indeed, the effect of a series of phosphorylation events of a protein is the generation of a train of spots in the two-dimensional gel that differ significantly in their pI and moderately in their $M_r$. In case of SAP90/PSD-95, there are in addition isoforms that differ in both pI and $M_r$ suggesting that changes such as palmitoylation and/or expression of alternatively spliced forms may occur. Such high degree of diversity of single scaffold proteins has not been previously reported, and this demonstrates the usefulness of a proteome approach displaying the global PSD protein content. The different (post-translationally modified) forms of individual proteins may imbue them with an enhanced potential for differential interactions with various partners (35) potentially in an activity dependent manner. Although the ICAT LC-MS/MS experiment could not detect the presence of various SAP90/PSD-95 isoforms, it does identify extra scaffold proteins including chapsyn-110, densin-180, maguin, SAP 97, shank, and SAP 102, all of which have been previously reported to be PSD proteins.

*The Signaling Proteins*—The activity-dependent interaction of the various signaling pathways in the dendritic spine is considered as the basis of neuroplasticity. Accordingly, enzymes involved in these pathways are detected in our PSD preparation. It has been demonstrated that CaMKII$\alpha$ is the major protein constituent of the PSD fraction (18). In the present study CaMKII$\alpha$ is detected as 6 distinct spots that differ slightly in $M_r$ and/or pI, suggesting that they may be differentially post-translationally modified. Indeed, differential phosphorylation of CaMKII is considered to be a key factor in the trafficking and functioning of this protein (36). In contrast to CaMKII$\alpha$, only a single form of calmodulin is detected. Further, a number of enzymes from other signaling pathways are present in the PSD fraction, such as the cyclic nucleotide-dependent signaling pathway including protein phosphatase, PKA, and phosphodiesterase 11A2 that catalyze the hydrolysis of both cAMP and cGMP (37); the phosphoinositide pathway including IP 3-kinase and PKC; the G protein-coupled receptor pathway including the G protein, regulator of G protein signaling 8, and GIPC which is a single PDZ domain containing protein that has been proposed to regulate G protein signaling (38). Neurofibromin is a ras GTPase-activating protein, which function as negative regulator of Ras. This protein is involved in the processes of neuronal development (39) and learning (40). SynGAP is another ras GTPase-activating protein that interacts with SAP90/PSD-95, and may regulate the MAP kinase pathway within the dendritic spine. Recent studies indicate that this protein modulates neuronal development, regulates glutamate receptor synaptic targeting, and is involved in the induction of LTP (41–42). Citron also interacts with SAP90/PSD-95 (43), and functions as Rho/Rac effector protein involved in the organization of the cytoskeleton (44). Wnt signaling pathway, which may be involved in the synaptic modeling and pre- and postsynaptic differentiation (45), possible exists in the PSD because we detect the Wnt inhibitor factor 1, which negatively regulates Wnt signaling (46). $\kappa$B-ras 1 is known to interact with NF-$\kappa$ and I$\kappa$-B and regulates their activity. Both NF-$\kappa$ and I$\kappa$-B have previously been detected as PSD proteins (47), and are implicated as molecules that mediate signaling to the cell body and modulate gene expression. Other signaling proteins are known to occur at a much lower level than for instance CaMKII in the PSD fraction, and some of them may also exist only in a subpopulation of the synapses. For example, nitric-oxide synthase was shown to be present in less than 10% of the synapses (48). Probably due to their low abundance, these proteins remain undetected in our study.

*The Cytoskeletal and Their Interacting Proteins*—The architectures of the PSD and the dendritic spines are maintained by a number of cytoskeletal proteins. In agreement with previous studies (18) we have identified a variety of these proteins, including actin, tubulin, plectin, internexin, neurofilament, spectrin, myosin, microtubule-associated protein, and tropomyosin.

Several cytoskeletal proteins are highly abundant in the PSD fraction, and they form a cytoskeletal matrix in the spine that gives the characteristic electron dense appearance of the PSD (21). Frequently, single protein species are detected as multiple protein spots on the two-dimensional gel, suggesting that isoforms or post-translational modifications of the proteins occur. In this respect, phosphorylation of cytoskeletal proteins (as driven by the changes in signal transduction events) plays an important role in their polymerization/depolymerization and the activity-dependent modulation of the cytoskeletal matrix (49–50).

The PSD and synaptic spines are not static; changes in neuronal activity can bring about rapid alteration of the size and morphology of these structures, which in turn may change the synaptic efficacy (51–52). Of particular interest is a recent study that demonstrated that 85% of actin in the spine is dynamic, with a turnover rate of 44 s (53). As actin microfilaments are associated with structural plasticity, the identification of a number of actin-regulatory proteins in the present study is entirely consistent with the idea of rapid structural changes driven by reorganization of the actin-microfilament in the spine. These proteins include cofilin, which depolymerizes actin filaments from its pointed end thereby increasing actin dynamics (54); F-actin capping protein Z and capping protein $\alpha$2, which bind to the barbed end of actin filaments (54); neurabin 2, which binds to actin and recruits protein phosphatase to actin that controls actin rearrangement (55); the LIM and SH3 domain-containing protein, which is proposed to play important roles in the regulation of dynamic actin-based, cytoskeletal activities (56). Another LIM domain containing protein present in the PSD fraction, the cysteine rich protein, has been shown to interact with the actin cytoskeleton and may be involved in muscle differentiation (57). Tropomodulin lowers the apparent affinity of pointed ends of the actin filament for actin monomers (58). Syndapin regulates endocytosis and actin dynamics (59). Chaperonin containing tail-less complex polypeptide 1 is known to assist folding of actin and tubulin (60). Insulin receptor tyrosine kinase substrate p58/53 interacts with the ProSAP/Shank scaffolding proteins of the PSD (61); in addition to its function as a substrate of insulin receptor signaling this protein is also known to regulate cytosketelal dynamics (62). Actin related protein 2/3 (54, 62), and related members together form the actin nucleation center for the polymerization of actin. Insulin receptor tyrosine kinase substrate p58/53 together with the members of Wiskott-Aldrich syndrome protein family are also involved in this process (62). In addition, we have identified some cytoskeletal protein-interacting proteins such as the neuronal protein 22, and the trans-

TABLE II
*Functional clustering of the identified proteins*

Novel proteins and proteins with described functions that cannot be grouped as below are not shown. Many proteins are detected as multiple spots on the two-dimensional gel, strongly suggesting the presence of post-translational modifications of the proteins and/or the expression of isoforms. Proteins identified from the ICAT experiment alone do not contain a spot number.

| Protein name | Spot number |
|---|---|
| Scaffold proteins | |
| SAP90/PSD-95 | 178; 179; 180; 222; 246; 247 |
| Vesl-1L/Homer 1 | 110; 129; 131; 135; 142; 143 |
| PSD-95-binding protein (GKAP/SAPAP1) | 222 |
| Chapsyn-110/PSD93 | |
| SAP102 | |
| SAP97 | |
| Maguin | |
| Shank-1 | |
| Densin-180 | |
| Signaling proteins | |
| CaMKII, $\alpha$ | 76; 77; 79; 80; 249; 250 |
| IP3 kinase | 79; 80 |
| Protein phosphatase 1 | 105 |
| $\kappa$ B-ras 1 | 82 |
| Calmodulin | 89 |
| Ser/Thr protein kinase P78 | 202 |
| G protein $\beta$ 2 subunit | 119 |
| RGS 19-interacting protein 1/GIPC | 111 |
| Regulator of G protein signalling 8 | |
| cAMP-dependent protein kinase | |
| Phosphodiesterase 11A2 | |
| Citron | |
| SynGAP-a | |
| CaMKII, $\beta$, $\delta$, $\gamma$ | |
| Neurofibromatosis type 1 | |
| Wnt inhibition factor 1 | |
| Protein kinase C $\gamma$ | |
| Cytoskeletal and their interacting proteins | |
| Actin, $\beta$ | 124; 126; 132; 133; 136–141 |
| Actin-like | 187 |
| Tubulin, $\alpha$ | 121–123; 153; 155–157; 159; 162; 163; 189; 196; 202 |
| Tubulin, $\beta$ | 105; 108; 116; 117; 120; 127; 144; 145; 148; 150; 152; 158; 182–184 194; 195; 200 |
| Tubulin, $\gamma$ | 243 |
| Internexin, $\alpha$ | 161; 162; 164–170; 181; 185 |
| Neurofilament, light polypeptide | 140; 142; 146–149; 151; 160; 173; 174 |
| Neurofilament, middle polypeptide | 177 |
| Neurofilament, triplet M protein | 130; 134; 233 |
| Spectrin, $\alpha$ II | 230–232; 240 |
| Tropomyosin | 85 |
| Annexin V | 112 |
| Cofilin | 7; 8 |
| F-actin-capping protein | 100; 101; 244 |
| Capping protein $\alpha$ 2 | 103 |
| Neurabin 2/Spinophilin | 229 |
| LIM and SH3 protein 1/Lasp-1 | 47; 109 |
| Cysteine-rich protein 1 | 14 |
| Tropomodulin | 141 |
| Syndapin-1 | 148 |
| Chaperonin-containing TCP1 | 200 |
| Insulin receptor substrate protein 53/IRSP 53 | 211; 249; 250 |
| Actin-related protein 2/3 | 81 |
| Neuronal protein 22 | 13 |
| TCTP | 218 |
| Microtubule-associated protein | |
| Myosin Va | |
| Drebrin | |
| Plectin | |
| Proteins involved in Trafficking | |
| Sorting nexin 3 | 6 |
| CDCrel-1 | 69; 114; 115 |
| Septin-6 | 79; 80; 201 |
| Peanut (Drosophilia)-like 1 | 70 |
| Rabaptin-5 | 229 |
| TOB-3 | 11; 14 |
| *N*-ethylmaleimide-sensitive factor | |
| Chaperones | |
| Heat shock protein J2 | 25 |
| Hsc-70 | 173–176 |
| Hsp-70 | 224 |
| Bag-2 | 98 |
| C terminus of Hsp70-interacting protein (CHIP) | 118 |

TABLE II—*continued*

| Protein name | Spot number |
|---|---|
| Local synaptic protein synthetic machinery | |
| Acidic ribosomal protein P0 | 106 |
| Heterogeneous nuclear ribonucleoprotein H′ | 190–193 |
| Poly(rC)-binding protein 1 | 56; 66; 67 |
| Purine rich element-binding protein A | 68; 113 |
| Enoyl-coenzyme A hydratase | 27 |
| Translation elongation factor 1 $\alpha$ 2 | 234 |
| Initiation factor 5A | 96 |
| Translation initiation factor 4E | 241 |
| 40 S ribosomal protein S24 | |
| Energy production/transfer systems | |
| Glyceraldehyde-3-phosphate dehydrogenase | 28; 40–44; 228 |
| Triosephosphate isomerase 1 | 21; 22; 24 |
| Phosphoglycerate mutase type B | 23 |
| Aldolase, fructose-bisphosphate | 51–56 |
| Creatine kinase, brain | 128; 130 |
| Adenylate kinase 1 | 12 |
| Adenylate kinase 5 | 172 |
| Ubiquitination system | |
| Ubiquitin-conjugating enzyme E2 | 4 |
| Proteasome 26 S subunit, ATPase 3 | 148 |
| Ubiquitin carboxyl-terminal hydrolase L1 | 90; 221 |
| Pre-synaptic proteins | |
| Synapsin I | 210–215; 236; 237; 248 |
| Synapsin II | 71–75; 78; 204–209; 226; 227 |
| SNAP25-interacting protein 30 | 104; 109 |
| Rabphilin 3A | |
| Bassoon | |
| Receptors, Ion channel, and adhesion molecules | |
| NMDA receptor 2A | |
| NMDA receptor 2B | |
| NMDA receptor 1 | |
| AMPA receptor B | |
| Voltage-gated sodium channel | |
| Calcium channel isoform | |
| Brevican | |
| Neurofascin | |
| Plakoglobin | |

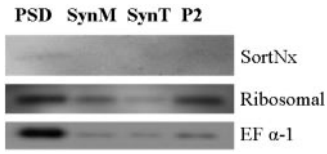


FIG. 4. **Immunoblots of representative proteins belonging to distinct functional groups from different cellular compartments.** Crude membrane fraction (*P2*), synaptosome (*SynT*), synaptic membrane (*SynM*), and PSD fraction were dissolved in lysis buffer, mixed with equal volume of 2× SDS buffer, and 10 $\mu$g each were resolved on a 10% SDS gel. After electroblotting to nitrocellulose membranes, the proteins were detected by an antibody against sorting nexin-3 (*SortNx*), serum of a patient with autoimmune disease against ribosomal proteins (*Ribosomal*) and an antibody against elongation factor 1 (*EF α-1*).

lationally controlled tumor protein. Annexin is shown to interact with actin. This calcium and phospholipid binding protein probably participates in membrane (re)organization and vesicle trafficking (63). Drebrin is a major actin-binding protein in the brain. Recently, it is reported that this protein is involved in the synaptic targeting of PSD-95 (64).

*Proteins Involved in Trafficking*—Various proteins travel into and out of the PSD, often in a neuronal activity-dependent manner. The alteration of the PSD protein content, for example the insertion or removal of glutamate receptors and SAP90/PSD-95, can modulate synaptic efficacy. Several proteins that are known to regulate vesicle trafficking are identified in the PSD preparation. For instance, sorting nexin 3 may interact with membrane receptors and target these to endosomes and/or recycle them back to the plasma membrane (65). Immunoblotting reveals that this protein is enriched in the PSD fraction. Two factors may underlie this enrichment; sorting nexin 3 may be embedded in the core structure of PSD or it may exist in the membranes of endosomes within the synaptic spine and are linked to PSD via cytoskeletal proteins.

CDCrel-1, peanut (Drosophila)-like 1, septin 6 and a hypothetical protein similar to septin, are GTPases that belong to the septin/CDC family. They may interact with actin, and may also self-assemble into a filament structure. They are implicated in the trafficking of vesicles and organizing proteins at the plasma membrane of a neuron (66). Previously, a septin (CDC 10) has been identified in a PSD fraction, and has been shown to be slightly enriched in the PSD (18). Rabaptin 5 forms a complex with other proteins such as Rab 5 that together regulate the trafficking of vesicles between the plasma membrane and an early endosome (67–68).

In the dendritic spine *N*-ethylmaleimide-sensitive factor maintains synaptic AMPA receptor response via the recycling of the receptors to the PSD and/or stabilizing AMPA receptors in the PSD membrane (69).

*The Chaperones*—Heat shock protein (Hsp) 70 kDa family molecular chaperones play critical roles in protein folding and trafficking. Previous studies revealed the presence of Hsc 70 and Hsp 40 in the PSD fraction (12, 18, 70). Similarly, we detect Hsc 70 and Hsp 40 in the PSD fraction. The heat shock proteins form a complex that is used for folding and conformational regulation of a variety of proteins, including receptors and signal transduction regulators.

In addition, two co-factors of the chaperones are identified, namely BAG-2 and C terminus of HSP 70 interaction protein (CHIP). BAG-2 may bind to the ATPase domain of Hsc 70, and inhibits the chaperone activity (71). Members of the BAG family were also shown to interact with other proteins, suggesting that they may operate as bridging molecules that recruit mo-

TABLE III

*Identified PSD proteins by ICAT nano-liquid chromatography tandem mass spectrometry*

The tandem mass spectra were searched against the NCBI database, using the Pro ICAT program. Proteins shown are present in the rat database. For those that are matched with two or more peptides, the minimum score is 10. For proteins identified with a single peptide the minimum confidence and score are set to 70 and 20, respectively. Number (Nr) refers to the number of peptides that are matched to a protein. For proteins that are matched with two or more peptides, the score and confidence of the highest value from a single peptide are shown. For database search one missed cleavage is allowed. In cases where the actual digested peptide and an extended partial with a missed cleavage are present, they are counted as distinct peptides.

| Accession no. | Name | Nr | Confidence | Score |
|---|---|---|---|---|
| gi\|294527 | 2′,3′-Cyclic nucleotide 3′-phosphodiesterase | 7 | 99 | 36 |
| gi\|11120682 | $Ca^{2+}$/calmodulin-dependent protein kinase II $\beta$ chain | 5 | 99 | 44 |
| gi\|10122138 | SynGAP-a | 4 | 99 | 49 |
| gi\|1095318 | SAP 97 | 4 | 99 | 40 |
| gi\|57429 | $\beta$-Tubulin T $\beta$ 15 | 4 | 99 | 34 |
| gi\|20879869 | Densin-180 | 4 | 99 | 32 |
| gi\|204402 | Glutamine synthetase | 4 | 99 | 28 |
| gi\|1864087 | SAPAP1 | 4 | 90 | 34 |
| gi\|27732033 | Creatine kinase, mitochondrial | 3 | 99 | 48 |
| gi\|6978593 | $Ca^{2+}$/calmodulin-dependent protein kinase II $\alpha$ chain | 3 | 99 | 42 |
| gi\|206455 | PSD-95/SAP 90 | 3 | 99 | 39 |
| gi\|27713216 | Tubulin $\alpha$ | 3 | 99 | 35 |
| gi\|1561642 | Plectin | 3 | 99 | 27 |
| gi\|6980984 | Glutamate receptor, NMDA2B | 3 | 99 | 21 |
| gi\|5081657 | *N*-Ethylmaleimide sensitive factor | 3 | 90 | 29 |
| gi\|27664478 | Fructose-bisphosphate aldolase A | 3 | 90 | 27 |
| gi\|125289 | $Ca^{2+}$/calmodulin-dependent protein kinase II $\gamma$ subunit | 3 | 75 | 26 |
| gi\|350611 | Transaminase, Glu oxaloacetic | 3 | 75 | 26 |
| gi\|2745840 | Postsynaptic density protein: citron | 3 | 70 | 20 |
| gi\|56198 | Glutamate dehydrogenase | 3 | 50 | 21 |
| gi\|8393206 | Cysteine-rich protein | 3 | 50 | 18 |
| gi\|1142640 | $\alpha$ actinin | 2 | 99 | 42 |
| gi\|56699 | Rat brain myelin phospholipid | 2 | 99 | 37 |
| gi\|205509 | Myelin/oligodendrocyte glycoprotein | 2 | 99 | 35 |
| gi\|461475 | ADP,ATP carrier protein | 2 | 99 | 31 |
| gi\|21245098 | Tubulin, $\beta$ 3 | 2 | 99 | 29 |
| gi\|11560113 | Chapsyn-110 | 2 | 99 | 25 |
| gi\|6978595 | $Ca^{2+}$/calmodulin-dependent protein kinase II delta chain | 2 | 90 | 33 |
| gi\|66727 | Protein kinase C $\gamma$ | 2 | 90 | 20 |
| gi\|6981264 | Neurofibromatosis type 1 | 2 | 75 | 20 |
| gi\|509397 | Aggrecan-like protein/ brevican | 2 | 70 | 14 |
| gi\|2155310 | Glutamate receptor, NMDA2A | 2 | 50 | 18 |
| gi\|13591886 | Microtubule-associated protein | 2 | 50 | 17 |
| gi\|9506427 | Bassoon | 2 | 50 | 16 |
| gi\|533711 | Rabphilin-3A | 2 | 50 | 14 |
| gi\|14029139 | Wnt inhibitor factor 1 | 2 | 40 | 18 |
| gi\|3087876 | Voltage-gated sodium channel variant | 2 | 40 | 17 |
| gi\|27683621 | Similar to actin $\gamma$ | 2 | 40 | 16 |
| gi\|27731499 | Similar to Zinc finger protein 208 | 2 | 30 | 18 |
| gi\|204382 | Glutamate receptor subunit 2 (GluR-b) | 2 | 25 | 12 |
| gi\|13431673 | Myosin Va | 2 | 10 | 14 |
| gi\|1346129 | Glial fibrillary acidic protein | 2 | 10 | 16 |
| gi\|14578563 | Calcium channel isoform $\alpha$ 1E7 | 2 | 10 | 11 |
| gi\|27684483 | Similar to class I $\beta$-tubulin | 2 | 10 | 12 |
| gi\|204964 | $\alpha$-Internexin | 1 | 99 | 40 |
| gi\|4761595 | Shank 1a | 1 | 99 | 38 |
| gi\|23592555 | Glyceraldehyde-3-phosphate dehydrogenase | 1 | 99 | 25 |
| gi\|13489065 | Maguin-2; maguin-1 | 1 | 95 | 22 |
| gi\|21728392 | Band 83 | 1 | 95 | 21 |
| gi\|1842427 | Neurofascin | 1 | 90 | 36 |
| gi\|9507161 | Synapsin 2 | 1 | 90 | 31 |
| gi\|27717199 | Protein kinase, cAMP-dependent regulatory, type II $\beta$ | 1 | 90 | 21 |
| gi\|1497985 | Plakoglobin | 1 | 75 | 26 |
| gi\|3452560 | Vesl-1L | 1 | 75 | 23 |
| gi\|2498314 | Drebrin A | 1 | 75 | 23 |
| gi\|27733035 | 40 S ribosomal protein S24 | 1 | 70 | 28 |
| gi\|236953 | SAP 102 | 1 | 70 | 24 |
| gi\|6093964 | Regulator of G protein signaling 8 | 1 | 70 | 24 |
| gi\|508971 | NMDAR1 glutamate receptor subunit | 1 | 70 | 21 |
| gi\|18143349 | Phosphodiesterase 11A2 | 1 | 70 | 20 |
| gi\|19705475 | Esau | 1 | 70 | 20 |

lecular chaperones to target proteins, presumably thereby modulating protein functions through alteration of the conformation.

CHIP decreases net ATPase activity of the chaperone and reduces their efficiency. This implicates CHIP in the negative regulation of the forward reaction of the Hsc70-Hsp70 substrate-binding cycle (72). CHIP also displays E3 ubiquitin ligase activity mediated by its U-box domain. CHIP can interact with BAG-1, and both proteins may be involved in the ubiquitination and proteasome-mediated degradation of proteins (73), including several membrane-bound receptors as well as Hsc 70, in a chaperone-dependent manner.

*The Local Synaptic Protein Synthetic Machinery*—Ribosomal proteins, and proteins that are involved in different stages of

translation process, were identified. The presence of these proteins in the PSD agrees with current studies on long term neuroplasticity demonstrating the requirement of the activity-dependent *de novo* protein synthesis in the synapses for the expression of long term potentiation and memory formation (74).

Immunoblot experiment shows that ribosomal protein is enriched in the PSD fraction. Previous electron microscopy revealed the neuronal activity-dependent trafficking of polyribosomes to the vicinity of the PSD, but polyribosomes are not embedded within the core structure of the PSD (75). Ribosomal proteins therefore most likely are linked physically to the PSD via the cytoskeletal proteins such as actin, which may explain the rapid vectorial trafficking of these ribosomes toward the PSD and also their localization close to the PSD (75).

Other proteins that may be involved in protein synthesis are also detected, namely heterogeneous nuclear ribonucleoprotein H, poly (rC) binding protein1, purine-rich element binding protein A, and enoyl-coenzyme A hydratase-like protein.

The eukaryotic elongation factor-1 $\alpha$ (EF-1$\alpha$) is prominently present in the preparation. This protein cooperates with other initiation factors, EF-1$\beta$, EF-1$\gamma$, and EF-1$\delta$ to induce efficient transfer of aminoacyl-tRNA to the ribosome. Initiation factor 5A is also characterized. This protein interacts with the 40 S initiation complex and causes the hydrolysis of ribosome-bound GTP. Finally, a low level of initiation factor 4 (IF-4) is detected. A recent study indicated the presence of IF-4 in the dendritic rafts and spines, and the protein was further shown to play an important role in synaptic protein synthesis (76).

Among the proteins that are involved in protein synthesis, EF-1$\alpha$ requires particular attention because it was reported that the concentration of EF-1$\alpha$ in some cellular systems greatly exceeds that of EF-1$\beta$, -$\gamma$, and -$\delta$, suggesting that it may have distinct functions independent of the other members of the elongation factor 1 family. Indeed, EF-1$\alpha$ has been shown to bind and modulate activities of diverse signaling proteins such as calmodulin (77), PLC-$\gamma$1 (78) and was shown to interact with muscarinic acetylcholine receptors (79). The role of EF-1$\alpha$ in the PSD has not been examined. Our immunoblot study reveals that EF-1$\alpha$ is highly enriched in the PSD preparation. This is in contrast to IF-4, which has been shown previously to be present throughout the dendrite (76). We propose that EF-1$\alpha$ may be implicated in signaling events of the PSD, in addition to its function in protein translation.

*The Energy Production/Transfer Systems*—Representative proteins from two classes of energy production/transfer systems were characterized, namely proteins involved in the glycolytic pathway, and proteins involved in the production and transfer of ATP (see also Refs. 12–13).

A number of enzymes involved in the glycolytic pathway are present in the PSD fraction, *i.e.* glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, phosphoglycerate mutase-1, and fructose-bisphosphate aldolase A. This is in agreement with a previous study, which demonstrated by immunoelectron microscopy the presence of glyceraldehyde-3-phosphate dehydrogenase in the dendritic spine and PSD (80). It was postulated that these proteins provide immediate availability of glycolytic source of ATP to the synapse. This is especially important during acute increase in synaptic activity when the mitochondrial supply of energy does not meet the transient and highly localized increased demand in energy.

In addition to the proteins involved in glycolysis, we detect proteins involved in energy generation, *i.e.* creatine kinase-B, and in the phosphate transfer system, *i.e.* adenylate kinases. These proteins play important roles in providing the site-specific burst of high energy phosphate (81). As these proteins are present in the PSD, they are strategically situated to accommodate the burst of ATP requirement during an increase in synaptic activity.

*The Ubiquitination System*—Previous studies indicated that many PSD proteins are ubiquitin-conjungated (82). As the ubiquitination/proteasome (Ub-Pr) pathway is a major route used to regulate many critical cellular proteins that must be rapidly destroyed (83), it was proposed that the Ub-Pr system regulates the synaptic protein composition and thereby also modulates functioning of the synapse (84). In line with this hypothesis, it was reported that the Ub-Pr pathway in part regulates the amount of syntaxin and G proteins, the endocytosis of glutamate receptor from the membrane, and seems to play a major role in regulation of synaptic plasticity (84–87). In addition, malfunctioning of the Ub-Pr system may underlie a number of degenerative disorders (83).

We have characterized several proteins from the PSD fractions that are part of the Ub-Pr system, namely a novel protein with high homology to the mouse ubiquitin-conjugating enzyme E2 that may play role in the conjugation of ubiquitin to the proteins to be targeted to the proteasome, the proteasome 26 S subunit. Also we found ATPase 3, which is an essential subunit of the core proteasome for the degradation of the ubiquitin tagged proteins, and the ubiquitin C-terminal hydrolase L1 which deubiquitinates proteins for recycling of free ubiquitin or to remove ubiquitin from incorrectly tagged proteins. The fact that many PSD proteins are ubiquitinated implies that ubiquitin-conjugating enzymes must be (transiently) present in the PSD. In this respect it may be biologically relevant that both BAG-2 and CHIP (a putative E3 ubiquitin ligase, 88) are present in the PSD as they might link the chaperone system to the ubiquitination system.

*The Receptors, Ion Channels, and Adhesion Proteins*—These classes of proteins are membrane bound and are identified mainly by the ICAT LC-MS/MS experiment. Glutamate receptors and ion channels are well described PSD constituents. Neurofascin belongs to the family of ankyrin-containing membrane-spanning cell adhesion molecules, and may link to the spectrin-based system for membrane-cytoskeletal connection (89). Brevican is a neural-specific chondroitin sulfate proteoglycan which functions as an extracellular matrix molecule, and may be involved in synaptic plasticity (90). Plakoglobin is similar to catenin $\gamma$. It is known that the cadherin-catenin complex is involved primarily in cell-cell adhesion and regulates the pre- and postsynaptic structures (91), and in addition may also regulate intracellular transduction pathways.

*The Presynaptic Proteins*—Some presynaptic proteins are recovered from the PSD preparation, as has been observed in previous studies (18). Similar to previous reports, synapsins are found in the PSD fraction. Synapsins may be co-purified with the PSD due to their intrinsic property to interact with high affinity to one of the major PSD proteins, namely CaMKII (92). Interestingly, we reveal that synapsins are represented by multiple spots *i.e.* 9 synapsin I and 14 synapsin II, suggesting the presence of alternatively spliced isoforms and/or differentially phosphorylated forms from single proteins. Furthermore, we detect the recently described SNAP 25 interacting protein 30 in two protein spots (93), and Rabphilin-3A.

*Concluding Remarks*—The present study reveals a high diversity of functional classes of protein in the PSD fraction. This is in agreement with previous electron microscopy studies that showed the possible extension of the PSD into the spine compartment; the spine apparatus, polyribosomes, and specialized endocytic zones are found located in close vicinity to the PSD that together with other "cytosolic" proteins such as glycolytic enzymes and signaling enzymes may be interconnected to the

PSD by actin filament (75, 94–97). Together, we postulate that the PSD is a complex organelle harboring diverse physiological functions, which puts the PSD into a central position for the autonomous functioning of the spine.

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